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Design, Synthesis and Characterization of Oriented Glyco-Affinity Macroligands for Glyco-Capturing, Glycomics and Glycoproteomics Applications

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DESIGN, SYNTHESIS AND CHARACTERIZATION OF ORIENTED GLYCO-AFFINITY MACROLIGANDS FOR GLYCO-CAPTURING, GLYCOMICS AND GLYCOPROTEOMICS APPLICATIONS

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DESIGN, SYNTHESIS AND CHARACTERIZATION OF ORIENTED GLYCO-AFFINITY MACROLIGANDS FOR GLYCO-CAPTURING, GLYCOMICS AND GLYCOPROTEOMICS APPLICATIONS

SRINIVAS CHALAGALLA

ABSTRACT

Cell surface carbohydrates existing as parts of glycoproteins, glycolipids, and other conjugates present the first information about cell to the outside world and are intimately involved in various biological events such as intercellular communication, and molecular and cellular targeting. However, mechanisms of most processes at the molecular level are still unclear. Therefore, it is very important to develop carbohydrate-specific binding molecules for rapid, efficient, sensitive purification and accurate analysis of complex carbohydrate structures as well as their functions. Furthermore, carbohydrate-specific binding molecules can be expected to be used in medical diagnostic applications for carbohydrate biomarkers. In this thesis study, oriented and multivalent carbohydrate-binding macromolecules were designed and developed based on a chain-end functionalized boronic acid-containing polymer (boropolymer) for glyco-capturing, glycomics and glycoproteomics applications. Namely, a biotin chain end and *O*-cyanate chain-end functionalized boropolymers were synthesized via aryalamine initiated cyanoxyl-mediated free radical polymerization in a one-pot fashion. The resultant boropolymers were characterized by ^{1}H-NMR and ^{13}C NMR spectroscopy. In our first study we demonstrated the efficient glyco-capturing followed by direct MALDI mass spectrometry identification of the captured carbohydrate by using magnetic beads

functionalized with the biotin boropolymer via streptavidin/biotin interaction. In our second study we demonstrated oriented and covalent immobilization of *O*-Cyanate chain-end functionalized boropolymer on to amine-modified solid surfaces and its specific glyco-capturing capacity by QCM and AFM techniques. We further studied the multivalent interactions of the immobilized *O*-Cyanate chain end functionalized boropolymer with five different carbohydrate conjugated AuNPs. Our studies showed that different carbohydrates have different binding constants. Furthermore, the multivalent binding between carbohydrates and boropolymer was reversible and allowed the regeneration of boropolymer surface for sequential capture and release of the carbohydrate analytes. This oriented multivalent glyco-affinity ligands could be used for efficient carbohydrate and glyco-conjugates purification and identification and thus is expected to constitute a core strategy of glycomics and glycoproteomics targeting glyco-protein.

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CHAPTER I

INTRODUCTION

1.1 Introduction

Carbohydrates are important bio-molecules, which exist as parts of glycoproteins, glycolipids, and other conjugates present the first information about cell to the outside world and are intimately involved in various biological events such as intercellular communication, and molecular and cellular targeting.[1] The most well known carbohydrate involved biological processes are host–pathogen interactions,[2] immune response,[3] fertilization,[4] transport of sugars across cell membranes[5] (Figure 1). However, mechanisms of most processes in molecular level are still unclear. In fact, development of molecular ligands that tightly and selectively bind to carbohydrates has proven to be very difficult. The difficulty is explained by conformational flexibility of the linkages between different units of carbohydrates, and very limited variety of functional groups to interact with. In addition, the quasi dominance of hydroxyl groups on the structure of carbohydrates makes it difficult for a ligand to compete with water when used as bulk solvent. Therefore, development of carbohydrate-binding molecules could be used for carbohydrates and glyco-conjugates purification and identification application. Subsequently, development of carbohydrate-binding molecules thus can be used for functional investigation of carbohydrates in physiological pathway and thus is expected to constitute a core strategy of glycomics and glycoproteomics targeting glycoproteins.

Furthermore, development of carbohydrate-binding molecules may offer control over pathological pathways and may have medical diagnostic applications as sensors for carbohydrate biomarkers. Other potential applications for such systems are numerous, including pharmaceutical delivery agents, biological probes for medical imaging, sensors for industrial monitoring of carbohydrate, and carbohydrate and glycoconjugate purification applications.

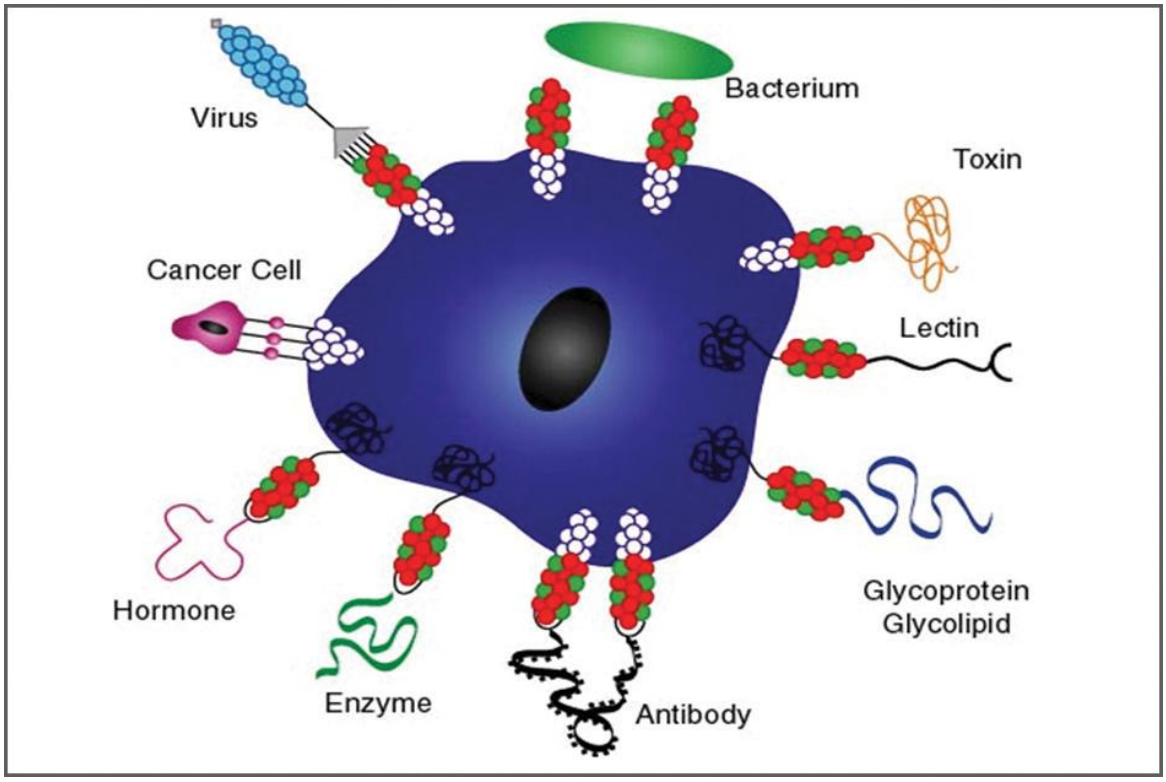


Figure 1. Cell surface carbohydrate-protein interactions in physiological and pathological processes. Figure is cited from Dr. Chun-Hung Lin web-page.

1.2. Popular methods for isolation& identification of carbohydrates and glyco-conjugates

1.2.1. Lectins: Carbohydrate binding proteins

Natural carbohydrate-binding molecules, such as lectins, are ubiquitous in the natural world and have been identified in animals, plants, bacteria, and viruses.[6] Traditionally, glycan-binding protein, lectins have been used to determine the glycan composition of cells or glycoproteins.[7] Recently, the use of lectins as a glycoprofiling tool has become sophisticated with the advent of lectin microarrays, in which panels of lectins are immobilized on a single chip for glycomic analysis.[8-12] However, most lectins are from natural sources, which can introduce some variability in their binding affinities dependent on purification. Some lectins can also be cross specific, binding multiple glycan structures[13]. Furthermore, lectin-based system lacks of stability and has short lifetime, thus require replenishment due to their intrinsic protein nature. Alternatively, several molecular architectures have been employed in the development of lectin-like carbohydrate receptors.

1.2.2. Hydrazide Chemistry

Compared with lectin affinity approach, the hydrazide chemistry enrichment of glycoproteins is based on covalent reaction, which results in less non-specific binding. Hydrazide functional groups can efficiently trap glycopeptides by covalent bindings after oxidation of cis-diol groups of carbohydrates with periodate[14,15]. The major deficiency is that the oxidation step would increase both experiment time and sample complexity whereas lectin affinity approach is relatively simple and flexible to use on single, combination or series of lectins. Outcome of the two methods was evaluated using human

cerebrospinal fluid regarding number of identification and capturing specificity.[16] Many glycopeptides were observed in lectin based affinity approach whereas the hydrazide chemistry method has higher specificity (81%) than the lectin method (69%).

1.2.3. Hydrophilic interaction liquid Chromatography & Size exclusion Chromatography

Based on the hydrophilicity of glycan moiety, hydrophilic-interaction liquid chromatography has also been used to enrich glycopeptides.[17] Since some glycopeptides have strong hydrophobic nature while some non-glycopeptides are highly hydrophilic giving rise to some false-positive and false-negative results. Alvarez-Manilla et al. demonstrated that the size-exclusion chromatography enriches *N*-linked glycopeptides relative to non-glycopeptides. This method is based on the fact that glycopeptides have increased mass relative to non-glycosylated peptides however, the co-enrichment of large non-glycopeptide (false positive) and missing of small glycopeptides (false negative) are the main disadvantages of this method.[18]

1.3. Boronic Acids as Ligand for *cis*-Diols

Reversible complex formation of borate and phenylboronate ions with diols in aqueous solution has been well known for many years.[19-21] These interactions were employed in separation[22-24] and detection[25-28] of sugars, glycoproteins,[29-31] nucleotides,[32] and nucleic acids[33] and synthesis of sugar-responsive materials.[34] Equilibrium constants of the complex formation (K_{ass}) have been reported, [19-21] including those relevant to borate interaction with particular diols in the saccharide molecules.[35] Values of the constants vary from 6 to 2000 M^{-1} for different sugars, [21] suggesting high selectivity of borate interaction with glycoproteins and carbohydrates bound to cells. Recognition of

carbohydrates by lectins and antibodies is involved in many important processes in nature, including cell-cell interaction,[36] adhesions of bacteria to animal tissues,[37] and others. One can suppose, therefore, those synthetic, sugar-sensitive polymers may act as effective regulators of the above bioadhesion phenomena. Recently, some efforts were made to synthesize water-soluble copolymers containing phenylboronic acid and to study their lectin-like interactions with cells.[38, 39] The selectivity of binding to the cell carbohydrates can be, however, different between the pendant and free phenylboronate, either due to multipoint chelation of carbohydrate residues by pendant phenylboronates or for any sterical reasons. Interaction of thermoresponsive boronate-containing copolymers with some carbohydrates and polyols was recently studied[40] in a comparative way: owing to the hydrophilic properties of the associating molecules, their binding to the copolymer led to a shift of its phase transition temperature (T_P),[40,41] which might be easily quantified.[40] Different reactivities of carbohydrates with phenylboronic acid immobilized on an insoluble carrier has also been demonstrated in a chromatographic mode.[30]

1.3.1. Boronic acid-contining macromolecules for carbohydrate characterization

In one of the earlier studies Kataoka[42] *et al* reported totally synthetic glucose responsive polymer gels. The major component of the matrix of this glucose-responsive gel is poly (N-isopropylacrylamide) (PNIPAAm) functionalized with a definite fraction of a 3-acrylamidophenylboronic acid (AAPBA) as the glucose- sensing moiety. A small amount of N,N′-methylene-bis-acrylamide (NMBA) was used as a cross-linker and 2,2′-azobis(2,4-dimethylvale- ronitrile) (1 mg/mL) as an initiator of radical polymerization. The study reports that phenylboronic acid compounds in aqueous solutions exist in

equilibrium between the undissociated (or uncharged) and the dissociated (or charged) forms. Because only charged borates can form a stable complex with glucose in aqueous solutions, the equilibrium can be shifted in the direction of increasing charged phenylborates through glucose complexation. Direct complexation of the uncharged form with glucose is known to be unstable in water because of its high susceptibility to hydrolysis. Consequently, an increase in glucose concentration of the solution increases the fraction of the total borate anions, decreasing the fraction of the uncharged form. As charged borates are more hydrophilic than the uncharged form, a glucose- dependent change in the ratio between those two borate forms in the polymer chain should crucially affect the polymer solubility, if the polymer chain has an amphiphilic character. And in this study, a sharp transition in the swelling degree of the gel system was achieved with glucose concentration and is temperature dependent. The group well exploited this property, and used it as a chemical valve to regulate solute permeation responding to glucose, in which the solute tested was insulin. Thus, the on-off regulation of insulin release from the gel was achieved through a drastic change in the solute transport property as a result of the formation and disruption of the surface barrier layer of the gel. Mattiasson[43,44] et al also used AAPBA to synthesize a copolymer of NIPAAm for sugar moiety detection and the resulting boropolymer was shown to thermoresponsive. In a separate study, Jackson[45] et al directly incorporated specialized carbohydrate affinity ligand methacrylamido phenylboronic acid (MAAPBA) in polyacrylamide gels for fluorophore-assisted carbohydrate electrophoresis. This boron affinity saccharide electrophoresis separation of the carbohydrates greatly improved the effective separation of saccharides that show similar mobilities in standard electrophoresis, eliminated

inverted parabolic migration, completely restored their predicted running order based on their charge/mass ratio, and resulted in improved separation of the analyte saccharides.

In another study, Kitano and Kanayama[46] reported using of boronic acid containing SAMs for the interfacial recognition of sugars. By coupling APBA with dithiodialiphatic acid using a water soluble carbodiimide, the disulfide with two boronic acid groups at ends were obtained, which were able to form self-assembled monolayers (SAMs) on both silver and gold colloids and on gold electrodes as well. Small sugars like fructose, glucose, mannose, galactose etc and polysaccharide mannan were shown to bound to the boronic acid SAM on the gold colloid, as detected by its absorption changes at 522 nm. The affinity order was determined to be fructose > mannose > galactose > glucose, and the results were attributed to the regioselective binding of the phenylboronic acid group in the SAM to hydroxyl groups of the sugars. Ivanov[47] *et al* by using APBA as ligand, evaluated boronate containing thin polyacrylamide gels (B-Gel), polymer brushes (B-Brush) and chemisorbed organosilane layers (B-COSL) as substrates for carbohydrate-mediated cell adhesion. These three types of substrates differed in surface microstructure and in three - dimensional organization of the affinity polymeric reagents. By using M2139 murine hybridoma cells, the authors showed that B-Brush substrate was superior with respect to cell adhesion and viability attributed this to the motional freedom of the end-grafted polymer chains on them. Interestingly, the adhered cells could be detached by using 0.1M fructose as a competitor of cell binding to the boronate-containing substrates, while preserving the cell viability and cell functions.

Kataoka[48] *et al* reported a novel phenylborate glucose responsive polymer at physiological pH. They used a newly synthesized phenylborate derivative 4-(1,6-dioxo-2,5-diaza-7-oxamyl) phenylboronic acid, (DDOPBA) and poly(N-isopropylmethacrylamide), (PNIPMAAm) as the main chain with critical solution behavior at closer physiological temperature ranges.

Ivanov[49] *et al* reported using boronate-containing copolymers (BCC) brushes on the siliceous supports for affinity adhesion of carbohydrate particles and yeast cells. Cross-linked agarose particles (Sepharose CL-6B) and baker's yeast cells were showed to adhere to these siliceous supports end-grafted with boropolymers (BCCs) of N, N-dimethylacrylamide (DMAA) at pH >7.49. In a separate experiment Ivanov and Mattiasson[50] reported comparative studies of BCC of N-acryloyl-m-aminophenylboronic acid (NAAPBA) with either DMAA or N-isopropylacrylamide (NIPAM) and it was shown that the binding of saccharides to BCC of NIPAM resulted in a shift of its phase transition temperature (ΔT_P), and this ΔT_P order for tested non-reducing ends of glycoproteins was reported to be N-acetylneuraminic acid > xylose ≈ galactose > mannose ≈ fucose >> N-acetylglucosamine.

Kurczewska and Schroeder [51] used formylphenylboronic acid modified silica surfaces as artificial receptors for sugars in aqueous solutions with ARS dye as the reporter of the sugar binding. Recently, Willner[52] et al reported selective and enantioselective analysis of mono and disaccharides using imprinted boronic acid-functionalized Au nanoparticle composites and surface plasmon resonance spectroscopy.

In another study Schoneich[53] *et al* reported a new type of boronate affinity phases containing immobilized sulfonamide- and sulfonyl-phenylboronic acids on porous silica material. The new phases showed an enhanced boronate affinity at neutral and slightly acidic pH and are particularly suitable for oxidation-sensitive analytes.

Numerous studies have focused on isolation and characterization of glycoprotein and glycopeptides. Because of their complex chemistry the literature is overwhelmed with a variety of data differing in their procedures. The most relevant studies which used polyboronates in one form or other will be discussed in this section.

In one of the earlier studies, Kim[54] et al used aminophenylboronic acid (APBA) immobilized magnetic beads for glycoprotein determination by matrix assisted laser desorption/ionizaton time of flight mass spectrometry (MALDI-TOF-MS). Specifically they studied glycoproteins, such as HbA1c, fibrinogen, and RNase B and suggested that by using a boropolymer on magnetic beads, it is possible to directly determine the glycoprotein by MALDI-TOF-MS. In a similar study, Deng[55] et al presented a simple approach to prepare polyboronate magnetic beads for glycoprotein capture. Specifically they used the APBA to functionalize the magnetic nano particles and MALDI-MS to determine the beads affinity for the glycoproteins. In another study, Kim[56] et al used APBA to prepare functionalized self-assembled monolayers (SAMs) of alkanethiols on gold. They designed a SAM based MALDI -TOF - MS plate (SAMDI), with a spacer group like oligo(ethylene glycol) (OEG), to reduce the nonspecific adsorption/adhesion and for direct detection of glycoproteins after affinity-capture (or enrichment) on the plate. The authors reported that SAMs of alkanethiols on gold presented hydroxyl groups with a background of methoxy- terminated tri (ethylene glycol) (EG) groups in a ratio of

1:2.The terminal hydroxyl groups of the SAMs were activated by N,N'- disuccinimidyl carbonate, followed by conjugation with aminophenylboronic acid to obtain APBA functionalized SAMDI plates. Further, the hydroxyl groups served as a handle for immobilizing APBA after activation and the OEG group contributed to the inertness of the monolayers thereby preventing the nonspecific adsorption of proteins. In another study Yang [57] et al also used APBA to functionalize composite nano particles for glycoprotein analysis. Composite nano-particles are specially synthesized nano-particles exploiting the advantages of both magnetic and gold nano particles. Thus composite nanoparticles have both the magnetic properties of superparamagnetic particles and the biocompatibility and surface chemistry of gold. The authors reported the fabrication of a core – satellite structured composite material, with a silica-coated ferrite "core" and "satellites" of gold nanoparticles on which the "anchors" of 3-aminophenylboronic acid (APBA) are also present for capturing glycosylated proteins/peptides. The authors concluded that these types of beads are highly specific and sensitive with a reported glycoprotein capturing (adsorption) capacity of more than 3 times when compared with that of commercial magnetic beads, on a gram-to-gram basis.

In another study, Chen[62] et al developed APBA self-assembled layer on glassy carbon electrodes (GCE), in which 3-aminophenylboronic acid (APBA) is covalently bonded to the electrochemically pretreated GCE surface with glutaraldehyde linkage. The specific binding of glycoprotein peroxidase with this self-assembled layer had been studied using horseradish peroxidase (HRP) and assays were performed using cyclic voltammetry, electrochemical impedance studies and photometric activity studies. The electrochemical

measurements and photometric results showed that approximately 70% of the bound HRP is specific binding.

Further to the above studies, which used APBA as a mono-functionalizing agent, Fernandez[61] et al showed that APBA could also be used in conjunction with other moieties. This group reported the use of mixed epoxy-boronic acid monolayer electrodes for peroxidase immobilization. The principle underlying their strategy was the increased immobilization rate achieved by the weak interaction-induced proximity effect between slow reacting oxyrane groups in the SAM and nucleophilic residues from adsorbed proteins, which allows the formation of very stable covalent bonds. Their approach was studied by the use of phenylboronates-oxyrane mixed SAMs, which combine the affinity motif of boronates with the reactivity of epoxy groups able to covalently link the protein to the SAMs. Interestingly, the APBA itself was bound to the monolayer through some of these epoxy groups, leaving the rest available for later interaction with the protein. These epoxy-boronate functional monolayer electrodes are versatile, stable interfaces, ready to incorporate glycoproteins by incubation under mild conditions.

Some of the recent studies have used 4-mercaptophenylboronic acid (MPBA) as a boropolymer for the glycoprotein detection. The Deng and Yao [58] group reported a study in which MPBA was used to functionalize the gold particles. In their work a stainless steel MALDI target plate was spotted and sintered with gold nanoparticles and functionalized with MPBA/ethanol solution during which MPBA is immobilized on the surface of gold nanoparticles through Au–S bonding. The gold spots on the plate served the dual role, both as a solid - phase extractor to enrich target molecules and as a stable MALDI matrix for the ionization of the retained molecules, which can be further

analyzed by MS. Interestingly, as the supporting substrate, gold can also adsorb UV laser to help desorption and ionization of trapped molecules, which provides effective and sensitive enrichment for analysis of glycopeptides from peptides mixtures. Since the boronic acid and cis-diol interaction is reversible, the authors were able to regenerate the plate by immersing in an acid solution containing 5% TFA and 20% ACN solution, once the analysis was complete. In a similar study by Deng and co-workers [59] used MPBA to functionalize the gold particles. Specifically, the authors used FTIR to substantiate the Au-S bond of 4-MPBA-gold nanoparticles that is further supported by UV/Vis adsorption spectra. Using these MPBA-modified gold nanoparticles, they performed the isolation and enrichment of glycopeptides in a standard protein (asialofetuin and horseradish peroxidase) digestion from a complex sample and the samples were further analysed by matrix-assisted laser desorption/ionization quadruple ion trap time- of-flight (MALDI-QIT-TOF) mass spectrometry. Similar to the above-mentioned study using APBA, Deng and Zhang [60] in a recent study showed the usefulness of MPBA on composite nanoparticles as well. In this study, initially the Fe_3O_4 - C magnetic microspheres were synthesized by the two-step solvothermal and hydrothermal reactions. Then, polyelectrolyte poly (diallyldimethylammonium chloride) (PDDA) was deposited onto the surface of Fe_3O_4 - C magnetic microspheres by electrostatic adsorption. The presence of an adsorbed layer of positively charged PDDA on Fe_3O_4 - C magnetic micro - spheres ensured the efficient adsorption of negatively charged gold nanoparticles. The self assembled Fe_3O_4 - C - Au magnetic micro-spheres function was further enhanced by modifying with 4-MPBA and the final composite nanospheres were used for selective enrichment of glycoproteins and glycopeptides.

Besides APBA and MPBA, Yoon and Song [63] directly used boronic acid itself to fabricate boronic acid-modified electrodes for electrochemical detection of glycated hemoglobin (HbA1c). To detect HbA1c, boronic acid was functionalized on the chemically modified electrode surface using poly (amidoamine) G4 dendrimer as an interface material. To confirm the HbA1c binding to the boronic acid layer, periodate-treated glucose oxidase (GO_x) was backfilled to the remaining amine groups of dendrimer on the electrode surface (in which, GO_x was selected to amplify the electrochemical signal by enzymatic catalysis) and quantified electrochemically. The results suggest that the boronic acid modified electrode has the potential to function as a biosensor for clinical diagnostics of glycoproteins such as HbA1c.

In another study by Liu [64] et al, the boropolymer was used to develop hydrophilic boronate affinity monolithic capillary for specific capture of glycoproteins by capillary liquid chromatography. The monolithic capillary was prepared by in situ free radical polymerization, using 4-vinylphenylboronic acid (VPBA) as functional monomer and N, N′ -methylenebisacrylamide (MBAA) as a cross-linker. In the synthesis of monolithic capillaries, reaction temperature and porogenic solution were two utmost factors of interest. According to the authors, the prepared capillary exhibited uniform open channel network and highly accessible ligand density. The prepared monolithic column was hydrophilic and biocompatible because of the hydrophilic cross-linker MBAA that also made it possible to capture glycoproteins from aqueous mobile phase. As the elution in boronate affinity chromatography is carried out with an acidic mobile phase such as

acetic or formic acid, nano-flow HPLC based on boronate affinity monolithic capillary has claimed to have very good compatibility with MS.

Polyboronates have also been shown to play a useful role in characterizing Amadori peptides. Frolov and Hoffmann [65] described a universal enrichment procedure for glycated peptides using boronic acid affinity chromatography in the first dimension and subsequently by reversed-phase chromatography, coupled either online to electrospray ionization mass spectrometry (ESI–MS) or offline to matrix-assisted laser desorption/ionization (MALDI) MS. The polyboronate used in this study was APBA.

In the recent studies, polyboronates have been employed along with other stationary phase affinity chromatography for glycoprotein characterization. Monzo, Bonn and Guttman [66] introduced a novel combination of boronic acid affinity chromatography with lectin affinity chromatography, which they termed as boronic acid–lectin affinity chromatography (BLAC). As boronic acid is considered as a pseudo-lectin that binds to cis-diol OH-containing structures, the choice of lectin as a co-stationary phase seemed to be appropriate. Concanavalin A (Con A) and wheat germ agglutinin (WGA) lectins were mixed with the pesudo-lectin boronic acid to form the BLAC affinity column and their performance was evaluated with standard glycoproteins. Specifically the authors used boronic acid agarose beads mixed with either agarose - bound Con A or agarose-bound WGA beads that supported selective and combined elution of standard glycoproteins with appropriate buffers viz; BLAC binding buffer, boronic acid elution buffer, concanavalin A elution buffer, wheat germ agglutinin elution buffer. The glycoproteins partitioned by this method can be further enriched to get comparative glycosylation profiling [67]. By using an automated affinity micropartitioning approach using combined boronic acid and

concanavalin A (BLAC/ Con A) resin-filled micropipette tips, human serum glycoproteins were isolated and enriched. The N-linked oligosaccharides of the partitioned glycoproteins were removed by PNGase F enzyme digestion, followed by 8-aminopyrene-1, 3, 6-trisulfonic acid labeling. Capillary gel electrophoresis with blue LED-induced fluorescence detection was applied in a multiplexed format for comparative glycan profiling. Using this technique, the authors compared normal human serum profiling with that of humans suffering from advanced prostrate cancer. Even though the capillary gel electrophoresis peaks are mostly identical for both, the prostrate cancer serum glycan profile showed four additional peaks, probably implying prostrate cancer specific glycosylation changes. In their further studies, the authors [68] showed that the elution from this BLAC is highest at 25^{o} C. The above studies show that boronates are of versatile use, provided the general experimental variables are optimized.

Recently some groups were reporting various approaches to synthesize the polyboronates. Linder[69] et al reported two inherently different approaches for the functionalization of polymethacrylate epoxy bare beads with boronate groups. In one method, the epoxy residues of the polymer particles were converted into reactive thiol groups which were subsequently used as anchor moieties for the immobilization of 4-vinyl - phenylboronic acid (VPBA) by radical addition or radical polymerization reaction. In another straightforward approach, the epoxy groups were reacted with 4-mercaptophenylboronic acid. According to the authors, functionalization of the thiol beads with 4-VPBA by radical addition reaction was at its best when carried out in aqueous acidic medium; therefore under these conditions the competing side reaction of esterification of the boronic acid can be avoided. Interestingly, nucleophilic substitution

of the epoxides at the surface of the polymethacrylate particles itself, with 4-MPBA turned out to be an equally efficient and straightforward approach for the synthesis of boropolymers. However, relevant data for the glycoprotein affinity of both boropolymers can't be readily located.

In another latest study, Ye[70] et al reported a novel approach to prepare boropolymer beads based on "Click chemistry". By introducing a terminal acetylene group into commercially available 3-aminophenyl boronic acids, a new "clickable" boronic acid ligand was synthesized. The clickable ligand, 3-(prop-2-ynyloxycarbonylamino) phenylboronic acid could be easily coupled to azide-functionalized hydrophilic Sepharose using Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction under mild condition. The resulting modified Sepharose beads, according to their work, showed most efficient separation of model glycoproteins and outperformed the existing commercially immobilized boronic acid gel.

Thus, from the above discussion, it is evident that polyboronates are one of the most versatile ligands that can be synthesized in a variety of ways and can be used in diverse settings to isolate and characterize complex glycoproteins and glycopeptides.

1.3.2. Boronic acid-contining macromolecules for catecholamine recognition

The boronic acid based compounds had been used to identify, analyze and quantify catecholamines including dopamine by employing various techniques. Dopamine is one of the most important members of its class playing a key role in different physiological and pathological processes. Given its important position, numerous studies have been focused on the analytical aspects of dopamine among its class members. Some of the

earlier works to detect dopamine used fluorescent chemosensors employing various dyes - for eg: diazapyrene based dyes, Lucifer yellow etc. In one study, Ali[71] *et al* used the later Lucifer yellow derivative - a water-soluble compound, with a couple of each sulfonate and carbonyl moieties on the naphthalene core (structurally similar to naphthalenedicarboximide) on a boropolymer, which they termed as 'compound 3'. This boropolymer produced maximum change in signal intensity with L-DOPA, the immediate *in vivo* dopamine precursor, among various related tested analytes. Even though multiple interactions were implied in this reaction the author(s) proved a boronic acid - aromatic diol interaction as one of them and concluded the 'compound 3' is a selective chemosensor for L-DOPA under physiological conditions.

In a similar study, B.Wang[72] *et al* on the basis of three-point recognition of dopamine *viz* boronic acid - dioil complexation, aromatic - hydrophobic interactions and a carboxylate - protonated amino ionic interaction; and computational chemistry studies, designed the first boronic acid based artificial receptors with alizarin Red S (ARS) dye as the reporter compound. The group successfully concluded that the series of boropolymers synthesized were highly selective to dopamine under near physiological conditions.

In another experiment, Glass[73] *et al* synthesized a boronic-acid derivative of coumarin aldehyde, which was shown to bind both dopamine and norepinephrine. It was postulated that an internal hydrogen bond is responsible for the colorimetric response to the catechol moiety. Interestingly, the compound in focus, which the author termed as 'sensor 2', had a differential fluroscense response, which was a decrease with catechol compounds and an increase with other tested amines. In a separate study of competitive color assay, Schrader [74] *et al* attempted to develop a method for detection of catecholamines in

complex samples like urine. For this, they synthesized a bezylamine receptor molecule that was derived from a combination of boronic acid with a bisphosphonate moiety, and of-course, with control on the spacers between them. Interestingly, they used the receptor precomplexed alizarin complexone from which an added competing catecholamine efficiently displaces the dye into the solution thereby producing a color change. Even though the assay is reliable, it was apparent that it needs optimization for selectivity. The boropolymers were also shown to be well effective in electrochemical detection of dopamine. Fabre and Taillebois[75], by applying a coat to an interdigitated microarray electrode (IMEs), used poly aniline boronic acid compound as receptor/sensor of the dopamine in conductometric experiments. One of the important features of this study was that the coated IMEs can be reused after immersing them in an acidic solution. Odashima[76] *et al* showed that a boronic acid derived hexahomotrioxacalix[3]arene polymer incorporated into PVC matrix liquid membranes can be used to detect dopamine in potentiometric studies. Shervedani and Bagherzadeh[77] successfully attached 4-Formylphenylboronic acid(BA) *in situ* on top of a gold cysteamine(CA) self-assembled monolayer electrode and the resulting Au-CA-BA matrix was shown to be a sensitive chemoselective sensor of dopamine. Yang[78] *et al,* for the first time, used boronic acid functionalized multi-walled carbon nanotubes (MWNTs) to develop a highly selective and sensitive electrochemical assay for dopamine recognition in the presence of excess L -ascorbic acid (L-AA).

Further to the above study, Willner[79] et al showed that a boronic acid-functionalized CdSe-ZnS semiconductor quantum dots (QDs) could be used to detect dopamine. They covalently linked 3-Aminophenyl boronic acid to glutathione (GSH)-capped CdSe–ZnS

QDs and applied the competitive analysis in which the change in fluorescence resonance energy transfer (FRET) when a ATTO-590 (dye) labeled dopamine was displaced by dopamine is measured. The binding of the dopamine to boronic acid receptor was shown to be a function of dopamine concentration itself.

Besides the assay development, membranes embedded boropolymers were also used in separation and/or purification processes of dopamine[80,81]. Thus the boroploymers are one of the important categories of compounds in dopamine recognition.

1.3.3. Boronic acid-contining macromolecules for nucleotide and RNA detection

Shinkai[82] et al reported boronic acid containing nucleotide responsive hydrogels. Functionalized hydrogels comprised of APBA as boronic acid monomer, (3-Acrylamidopropyl) trimethylammonium chloride as a cationic monomer, and N, N'-methylenebisacrylamide as a crosslinker monomer were prepared by radical copolymerization. These hydrogels were shown to efficiently bind nucleotides like AMP and ATP by both the boronic acid - cis-diol complexation and the electrostatic interaction between the cationic monomer and the nucleotide phosphate group and the binding process can be monitored by the nucleotide induced swelling and de-swelling of hydrogels in aqueous solution. Tuncel[83] et al also reported using APBA carrying-gel beads for nucleotide isolation. They used one of the hydrophilic monomers *viz;* either 2-hydroxypropylmethacrylate (HPMA) or polyethyleneglycol methacrylate (PEGMA) along with epoxypropyl methacrylate (EPMA) and by using ethyleneglycol dimethacrylate (EGDMA) as the crosslinker, the gel beads were obtained by the suspension copolymerization process. The resulting gel beads were named either HPMA

gel beads or PEGMA gel beads, which had different surface structures. Subsequently, the gel beads were functionalized using APBA via the direct chemical reaction between epoxypropyl and amino groups. By using β-nicotinamide adenine dinucleotide (β-NAD) the sorbent gel beads efficiency was demonstrated. In a similar study Cicek[84] reported using APBA as the ligand to modify hydrogel beads. However, the functionalization of the beads was achieved in a different way. The carboxyl groups on the gel bead surface were activated with a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) followed by covalent attachment of APBA via the amine groups. The nucleotide isolation ability of resulting sorbent beads was studied by β-NAD. In another study, the APBA was successfully used to functionalize thermosensitive copolymers. Tuncel[85] et al reported covalently attaching APBA to the thermosensitive copolymers of N-isopropylacrylamide (NIPA) and N-acryloxysuccinimide (NASI) via chemical reaction between succinimide and primary amine groups. They also reported that the resulting APBA-carrying NIPA–NASI copolymer showed both thermosensitive and pH-responsive properties, when investigated using RNA.

In some other studies 4-vinylphenylboronic acid (VPBA) had been used as functionalizing agent. Tuncel and Ozdemir[86] reported the synthesis of a gel matrix that was produced by the radical copolymerization of 2-hydroxyethylmethacrylate (HEMA) with VPBA. When used with potassium persulfate and tetramethylethylenediamine as the redox system and methylenebisacrylamide (MBA) as the crosslinker, the gel can be synthesized at low temperature (4°C). They also added either N-3-(dimethylamino)propylmethacrylamide (DMAPM) or 2-(dimethyl-amino)ethylmethacrylate (DMAEM) as amine-containing monomers to increase the diol

binding affinity of boronic acid gels. The resulting HEMA–VPBA–DMAPM or HEMA–VPBA–DMAEM terpolymer gels were studied using β-NAD. The same group, in other separate studies reported use of VPBA in thermosensitive gel matrix preparation of NIPAM[87] and also in thermosensitive N-isopropylacrylamide-4-vinylphenylboronic acid copolymer latex, poly (NIPA-co-VPBA) particles as well. In the later case, the use of boronic acid carrying sorbent in the thermosensitive form allowed the desorption of studied nucleotide - β-NAD, in a relatively wide pH range without much influence on the yield. In further work, Tuncel and Tuncel[88] successfully used VPBA to functionalize latex particles. The particles were produced by a ‘‘multi-step microsuspension polymerization’’ in which, boronic acid functionality was directly incorporated onto the macroporous particles during the polymerization by using a boronic acid-carrying monomer, to form a poly (styrene– vinylphenyl boronic acid–divinylbenzene) terpolymer and were tested as a sorbent for the adsorption of nucleotide β-NAD. This study specifically showed that, unlike the plain poly (styrene-co-divinylbenzene) particles, a four-fold higher β-NAD adsorption could be obtained with the boronic acid functionalized particles. Also, when used as a stationary phase in affinity-HPLC methods, the particles provided high-resolution liquid chromatograms secondary to the regular flow regime in the columns.

The VPBA was also used as ligand for RNA separation as well. Senel[89] reported the synthesis of an affinity membrane for isolation of RNA from nucleic acid mixtures. It was synthesized by the radical copolymerization of HEMA with VPBA. Potassium persulfate (KPS) and N,N,N’,N’-tetramethylethylenediamine (TEMED) were used as redox initiators and N,N-methylenebisacrylamide (MBA) was the crosslinker for

copolymerization at room temperature (22^{o} C). The authors showed that the RNA adsorption capacity of high VPBA containing membranes was almost double when compared with that of low VPBA membranes. In another study Tuncel[90] et al reported the synthesis of a random NIPA-VPBA copolymer with temperature and pH sensitivity for RNA characterization in aqueous media. These random copolymers of NIPA and VPBA were obtained by solution polymerization using 2,2'-azobisizobutyronitrile as the initiator in ethanol at 65^{o}C. In a further separate study, Tuncel[91] et al synthesized thermosensitive NIPA-co-VPBA copolymer latex particles that are even pH independent for RNA desorption.

1.4. Analytical Techniques used for Carbohydrate Capturing, Sensoring and Characterization in this study

1.4.1. Mass spectrometry

Mass spectrometry (MS) is a central tool used for monitoring, identifying and characterizing biomolecules in bioscience. With an increasing ability to correctly characterize miniscule quantities and more complex mixtures of proteins and peptides, MS is quickly becoming a key tool in the discovery of biomarkers. Additionally, because of its low detection limit and its ability for high throughput analyses, the possibility of using MS in clinical assays is also becoming a reality. For example, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is a promising high-throughput new approach for identifying new potential biomakers in various body fluids[92-97]. Recently, comparative analyses of glycans for biomarker discovery were performed with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS)

because of its ease of operation and high-throughput performance[98-100] Nevertheless, direct mass-spectrometric detection of biomarkers from complex sources such as blood serum/plasma, urine, and spinal fluid is quite difficult due to saline contaminants disturbing the MS ionization process. Furthermore, highly abundant proteins (human albumin in blood serum) interfere with the mass spectrometric analysis. Therefore, enrichment and purification are essential prior to MS analysis and the subsequent identification and structure elucidation.

1.4.2. Magnetic beads assistant affinity purification facilitates efficient proteomics of biomarkers

Magnetic beads-based bio separation is an emerging technology in biology and biochemistry. It has been widely used for the selective purification of proteins, nucleic acids, carbohydrate, cell organelles, and cells, directly from crude samples. Magnetic beads functionalized by different reactive groups, such as amine, carboxy, epoxy, tosyl, and affinity ligands such as streptavidin, protein A, IgG, etc., are commercially available. In general, the selection and isolation of cell and biomolecules have focused largely on the specificity of antibodies for cell surface ligands. Recently, immobilized ligands such as carbohydrates that exhibit a surface receptor-binding specificity have been employed for the rapid isolation of a variety of lectin-bearing cells and biomolecules. Rye and Bovin have demonstrated that glycopolymer-derivatized magnetic beads provide a useful tool for the selection of cells expressing a specific carbohydrate-binding phenotype[101,102]. Given the potential significance of this strategy, it is believed that the biocapture potential of carbohydrate derivatized magnetic beads can be significantly enhanced by immobilization of glycopolymers via a single chain terminus, which facilitates a uniform

and high oriented display of multivalent carbohydrate. Recently, our group has demonstrated the potential value of site-specific functionalized multivalent carbohydrate polymers combined with magnetic beads in bioseparation of carbohydrate-binding proteins[103]. Most recently, Sparbier et al. demonstrated the binding specificity of differently functionalized magnetic particles such as Concavalin A (Con A), boronic acid and Wheat germ agglutinin (WGA) to glycosylated peptides and proteins[104].

1.4.3. QCM Technique

The quartz crystal microbalance (QCM) technique has been identified as a highly powerful means to monitor adsorption/binding events to surfaces, and is being increasingly applied in bio recognition analysis[105-107]. The QCM is a piezoelectric mass-sensing device that works by sending an electrical signal through a gold-plated quartz crystal, which vibrates at a certain resonant frequency. The mass change on the crystal surface is monitored in terms of the change in resonant frequency of the crystal, based on the *Sauerbrey Equation*[108]*:*

$$\Delta f = -2f_0^2 mn/(\rho\mu)^{0.5} = -C_f \Delta m$$

Δf - Frequency Change (Hz) $\Delta \mathbf{m}$ - Mass Change (g)

ρ - Density of Qaurtz (2.648 g/cm^2) μ - Shear Modulus of Quartz

f_0 - Resonant Frequency (Hz)

The technique offers label free binding measurements in real-time, with high sensitivity and reliability. Carbohydrate recognition has only relatively been targeted recently with

QCM methods[109-113] showing that these systems are well suited for such analysis. Moreover, the QCM detection principle provides additional information about the structural and viscoelastic properties of adsorbed molecules[114]. QCM techniques are reliable, sensitive, and label-free, but they also have complicating factors associated with them. QCM requires expensive equipment and because the process cannot be miniaturized. The detection method can also be tedious and difficult at times due to problems with interference from non-specific binding events and/or background noise interference from stray electronic signals.

1.4.4. AFM Technique

Atomic force microscopy (AFM) is another useful technique that has been exploited by various research groups in protein imaging. AFM has been used to examine the details of molecular structures with exceptional resolution and without the need for rigorous sample preparation or labeling. AFM delivers topographical information at the nanometer scale. In AFM, a sharp nanometer-scale tip attached to the end of a cantilever rasters across a given area while a laser and a photodiode are used to monitor the tip deflections as a result of the force experienced from the sample. A feedback loop between the photodiode and a piezo-crystal corrects and records tip deflections according to the scanning parameters set[115,116]. The AFM can be used in two modes: contact mode imaging and intermittent contact mode. In contact mode, the cantilever tip maintains a constant force throughout the scan, while constant amplitude is maintained during intermittent contact mode imaging. Both of these imaging modes have practical applications, depending upon the surface to be scanned and the features one is trying to detect. The exceptionally low signal-to-noise ratio of the AFM allows individual biomolecules to be imaged at

nanometer resolution, presenting the opportunity to study the functionality of biomolecular assemblies. In addition to high-resolution capability, AFM can be used to directly measure intermolecular and intra-molecular interactions at the molecular level in order to acquire detailed insights about the function and structure of many biomolecular systems[115]. The resolution of topographical imaging has been tremendously improved mainly due to continuous developments of AFM instrumentation.

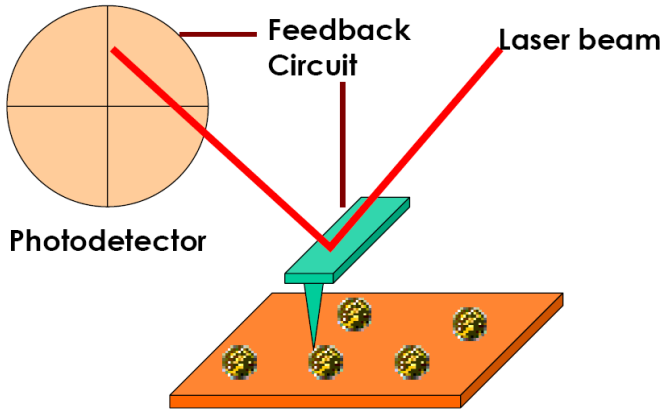
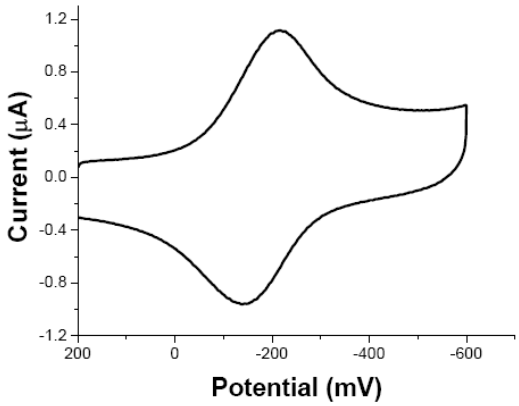


Figure 2. Schematic representation of an Atomic Force Microscope

1.4.5. Electrochemical techniques used in this study

Various electrochemical techniques will be used in our biosensor development studies, including cyclic voltammetry (CV), These methods were chosen because of their simplicity, sensitivity, cost effectiveness, and ability to gather much information within a few potential cycles. In CV, voltage (potential) is first decreased as a function of time to induce reductions. A current results as electrons are transferred to or from the analyte when the electrode reaches a specific voltage that matches the standard redox potential of the species in the cell. This is the reduction peak. Voltage is then increased back to allow oxidations to take place at the electrode, again when the potential matches the electron

transfer potential of the analyte. The resulting current from electron transfer makes an oxidation peak. Current is measured as a function of applied potential at a given scan rate, and the resultant data is represented by a curve that is called a voltamogram[117].



A typical Cyclic Voltammogram

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CHAPTER II

SYNTHESIS AND CHARACTERIZATION OF BIOTIN CHAIN-END FUNCTIONALIZED BORONIC ACID-CONTAINING POLYMER (BOROPOLYMER) AS FUNCTIONAL GLYCO-AFFINITY MACROLIGAND

2.1 Introduction

Boronic acid-containing compounds, which use covalent interactions, have been explored as artificial carbohydrate receptors [1], sensors [2], membrane transport agents [3], and cell surface carbohydrate recognition ligands [4], and as protective agents in synthesis of carbohydrates as well[5]. The broad interest in boronic acid-containing compounds results from their unique interactions with diols: they form cyclic esters with diols in water much more readily than many other acids. Recently, multivalent boronic acid-based polyol binders such as boronic acid-containing polymers have been developed for enhanced substrate affinity6-8. On the other hand, chain-end functionalized polymer facilitates site-specific immobilization required for generating uniformly oriented multivalent ligands [9-12].

In our study, we developed a biotin chain-end functionalized boronic acid-containing polymer (biotin boropolymer) as functional glyco-affinity macroligand for efficient purification and identification of carbohydrate and glycoconjugate. The biotin boropolymer was synthesized *via* a biotin derivatived arylamine initiated cyanoxyl-

mediated free-radical polymerization in one-pot fashion. The specific streptavidin binding capacity of biotin boropolymer was confirmed by streptavidin-HABA assay, while the specific carbohydrate binding capacity of biotin boropolymer was evaluated by Alizarin Red S binding assay. We then demonstrated a strategy for oriented immobilization of boropolymer onto magnetic bead surface through specific streptavidin-biotin binding and its glyco-affinity capturing followed by direct MALDI-MS identification of captured carbohydrate. (Figure 1).

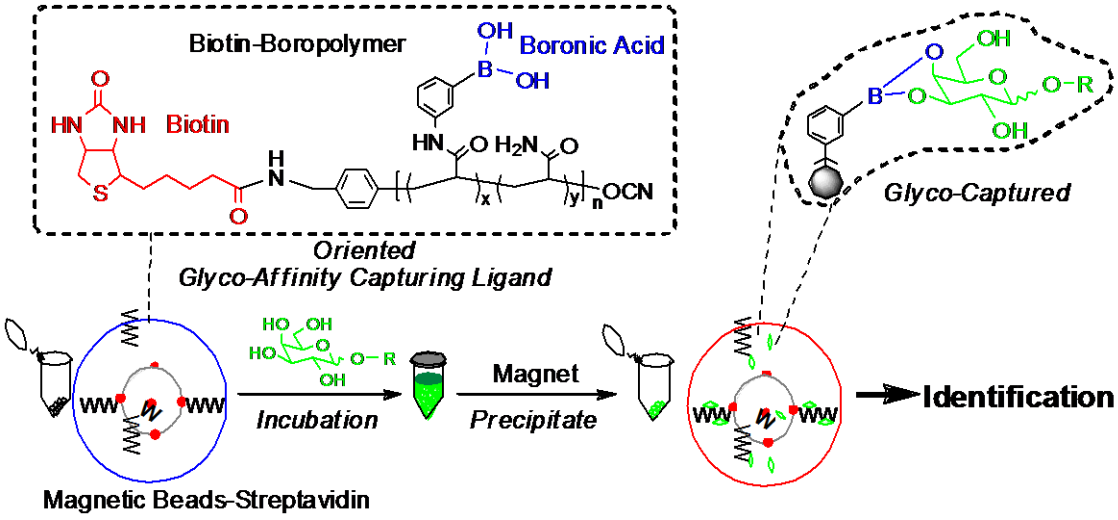


Fig. 1 Structure of biotin boropolymer as functional glyco-affinity capturing ligand for efficient carbohydrate and glycoconjugate purification and identification application combined with magnetic beads.

2.2 Experimental

2.2.1 Materials

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all polymerization reactions.

2.2.2. Methods

Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 µm thicknesses on which spots were visualized with UV light or by charring the plate after dipping in 10% H_2SO_4 in methanol. Mass spectra (MALDI-MS) were obtained using a Micromass Tofspec-2ETM instrument. ^{1}H NMR spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard.

2.2.3. Synthesis of biotinylated boropolymer

4-Aminobenzyl biotinamide **1** (55 mg, 0.15 mmol) and sodium nitrite (13 mg, 0.19 mmol) were dissolved in 2 mL of a mixture of water and THF (1:1) in a three necked flask. To the mixture, 0.41 mL of HBF_4 (48 wt%, 2.25 mmol) was added and allow it to react for 30 min at 0°C under Ar atmosphere. Next, a degassed mixture of 3-acrylaaminophenyl boronic acid **3** (36 mg, 0.19 mmol) and acrylamide (210 mg, 2.95 mmol) and sodium cyanate (50 mg, 0.77 mmol) dissolved in 1 mL of H_2O-THF (1:1) was introduced into the reaction flask above. The polymerization medium was heated to 50°C for about 16 hrs. Then the reaction mixture was cooled down to room temperature and THF was removed *via* evaporation under vacuum. The water solution was dialyzed

(3,500 mw cutoff) exhaustively against demonized water for about 36 hrs. Finally, the water solution was lyophilized to give the biotin-boropolymer **6** (200 mg, 55.1% conversion yield). The Cl-boropolymer **7** was synthesized similarly by using 4-chloro-analine **2** as an initiator in same mol ratios.

2.2.4. Streptavidin binding assay of biotin boropolymer

4-Hydroxyazo-benzene-2-carboxylic acid (AHBA) (0.012 mg, 5 x 10^{-5} mmol) dissolved in PBS (pH 7.4) (0.2 mL) was incubated with streptavidin (0.75 mg, 1.25 x 10^{-5} mmol) at room temperature for two hours. Next, UV-vis spectroscopy was taken before and after adding free biotin (0.012 mg, 5 x 10^{-5} mmol), biotin boropolymer **6** (0.25 mg, 5 x 10^{-5} mmol) and Cl-boropolymer **7** (0.25 mg, 5 x 10^{-5} mmol), respectively. All spectra were acquired on a Cary 50 Bio UV-visible spectrophotometer (Varian) at room temperature.

2.2.5. Alizarin Red S binding assay of biotin boropolymer

Alizarin Red S (0.072 mg, 3 x 10^{-4} mmol) dissolved in PBS (pH 7.4) (3 mL, 1 x 10^{-4} M) was incubated with biotin boropolymer **6** (4.5 mg, 9 x 10^{-4} mmol, 3 x 10^{-4} M) at room temperature for two hours. Next, UV-vis spectroscopy was taken before and after adding free galactose (100 mg, 0.55 mmol) in PBS (pH 7.4) (3 mL, 0.2.0 M), respectively. All spectra were acquired on a Cary 50 Bio UV-visible spectrophotometer (Varian) at room temperature.

2.2.6. Magnetic assistant glyco-capturing assay of biotin boropolymer

First, biotin end-terminated boropolymer **6** was immobilized onto the surface of streptavidin-derivatized magnetic beads (streptavidin C1 magnetic beads from Invitrogen) by incubating **6** (0.5 mg, 9.0 x 10^{-5} mmol) with magnetic beads (5 mg) in 0.5

mL of PBS (pH 7.4), followed by precipitation and thorough washing with PBS (pH 7.4). Next, the boropolymer-functionalized beads were incubated with lactose (0.5 mg, 1.5 x 10^{-3} mmol) as model carbohydrate in 0.5 mL of PBS (pH 8.3). Then the lactose-capturing magnetic beads were precipitated out and washed with PBS (pH 8.3) for three times to remove the unbound lactose.

2.2.7. Direct MALDI-MS identification of captured carbohydrate on magnetic beads

The lactose captured magnetic beads obtained above was mixed with 100 μL of the 2,5-dihydroxybenzoic acid (2,5-DHB) matrix (6 mg/mL in acetonitrile-0.1% TFA (2:1)), then the magnetic beads/matrix mixture was placed onto the MALDI plate for direct MALDI MS identification. Mass spectra were acquired in the linear positive mode.

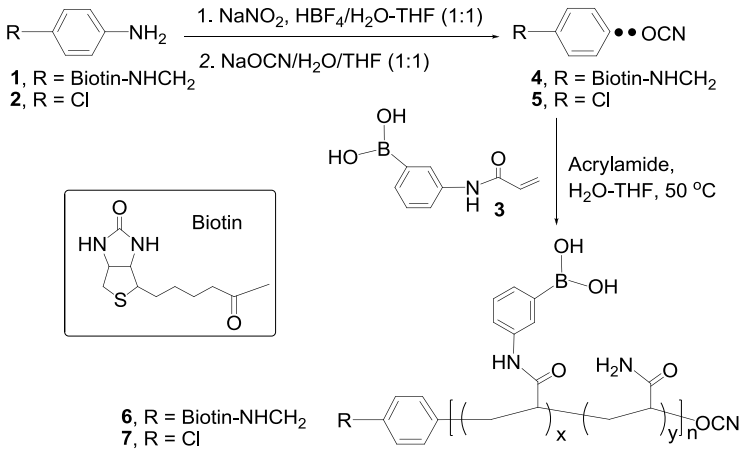
2.3. Results and Discussion

The desire to generate polymers with affinity ligand units pending on the polymer backbone and a functional group at the polymer chain end poses great synthetic challenges, including a requirement for serial protection-deprotection steps or further polymer derivatization after initial synthesis. In our previous study, we have demonstrated that arylamine initiated cyanoxyl-mediated free radical polymerization (CFRP) to be a straightforward approach to synthesize chain end functionalized glycopolymers[13-15]. Here, CFRP of acrylated phenylboronic acid by using biotin derivatived arylamine as initiator was investigated for preparing a biotin chain-end functionalized boropolymer. Briefly, cyanoxyl radicals were generated by an electron-transfer reaction between cyanate anions from a sodium cyanate aqueous solution and aryl-diazonium salts prepared *in situ* through a diazotization reaction of arylamine in

water. In addition to cyanoxyl persistent radicals, aryl-type active radicals were simultaneously produced, and only the latter species were capable of initiating polymer chain growth to afford biotin chain-end functionalized boropolymer **6** (Scheme 1). Similarly, a chlorine chain-end functionalized boropolymer **7** was synthesized by using 4-chloro-analine as an initiator as a control polymer.

2.3.1. Charecterization of boropolymers by ^{1}H-NMR spectroscopy

The resultant boropolymers were characterized by ^{1}H-NMR spectroscopy. As shown in Figure 2A, proton signal at 4.38 ppm, which was assigned for the biotin group and that at 7.00 ppm, which was assigned for the phenyl protons connecting biotin and polymer backbone with two methylene groups at *para*-position were the proof for existing of chain-end biotin group in boropolymer **6**. It is noteworthy that the phenyl group at the end of the boropolymer provides a convenient mechanism for calculating boronic acid content and the molar mass through comparison of integrated phenyl proton peaks with those of backbone protons. Comparison of the integrated signal from the phenyl protons (7.00 - 7.80 ppm) with that due to the polymer backbone methine (2.20 ppm) and methylene (1.60 ppm) indicated an average polymer composition of 5 phenylboronic acid and 70 acrylamide units for booropolymer **6** and **7,** respectively (Figure 2). Accordingly, the average molecular weights were 6,100 and 5,900 for booropolymer **6** and **7,** respectively.



Scheme 1. *One-pot* syntheses of biotin and chlorine chain-end functionalized boropolymers

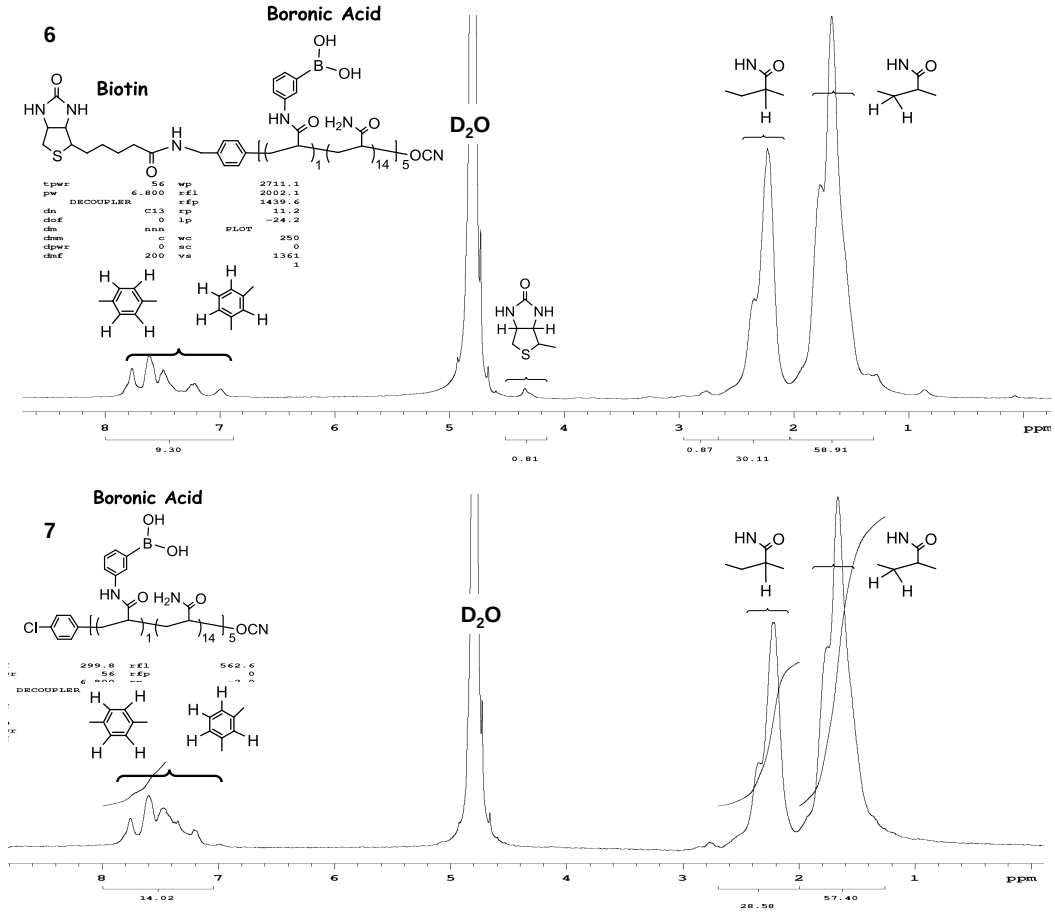


Fig. 2 ^{1}H NMR spectra of boropolymers in D_2O.

2.3.2. Streptavidin-HABA assay of biotin boropolymer

The specific streptavidin binding activity of the chain end biotin in biotin boropolymer **6** was accessed by using streptavidin-HABA (4'-hydroxyazo-benzene-2-carboxylic acid) assay [16]. HABA (λ_{max} 350 nm) changed color from yellow to red (λ_{max} 500 nm) upon binding to streptavidin (Figure 3, trial A), while biotin boropolymer **6** replaced the HABA out from its streptavidin complex as biotin-boronic acid and free biotin did (Figure 3, trial B, C, and D), but chlorine end-terminated boropolymer **7** did not (Figure 3, trial E) as monitored with UV spectroscopy. This result indicated the specific streptavidin binding capacity of biotin-boropolymer **6**.

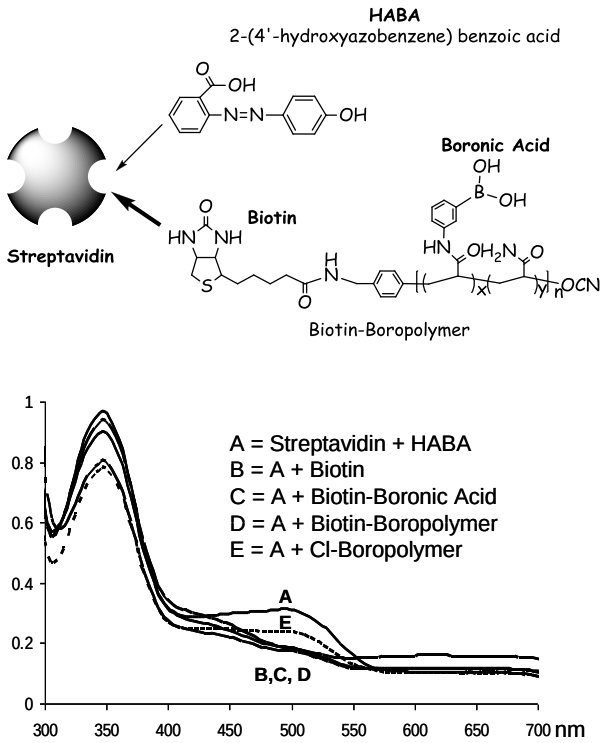


Fig. 3 Streptavidin-HABA assay of biotin boropolymer. All reactions were performed in PBS (pH 7.4) buffer at room temperature and were monitored by UV-vis spectroscopy.

2.3.3. Boronic acid/ ARS-sugar specific binding assay of biotin-boropolymer

Next, the specific carbohydrate binding capacity of biotin boropolymer **6** was evaluated by Alizarin Red S-binding assay. Specifically, Alizarin Red S (ARS) displays a dramatic change in fluorescence intensity and color in response to binding boronic acid and has been used as a general reporter for studying carbohydrate–boronic acid binding interactions [17]. In the present study, the effect of boropolymer on the spectroscopic property upon binding with ARS first and carbohydrate later in PBS (pH 7.4) buffer solution was examined. As shown in Figure 4, ARS showed a color change from deep red (Figure 4, trial A) to yellow (Figure 4, trial A + B) when binding to boropolymer in PBS (pH 7.4) buffer solution as monitored by UV absorption. Then, galactose used as a model carbohydrate to determine the binding between boropolymer and carbohydrates was added, the galactose–boropolymer complex formed immediately to release ARS out from its bropolymer complex, showing corresponding color change from yellow to red (Figure 4, trial A + B + C) as monitored by UV absorption. This result demonstrated the specific carbohydrate binding capacity of biotin-boropolymer **6**.

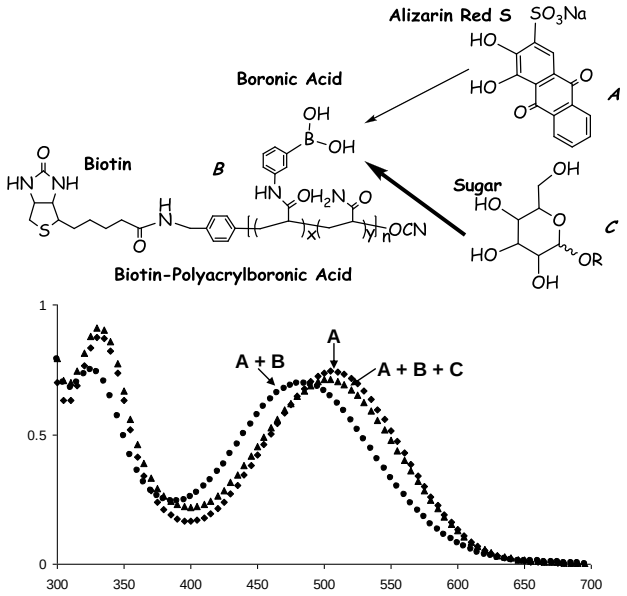


Fig. 4 Boronic acid/ ARS-sugar specific binding assay of biotin-boropolymer **6** as monitored by UV-vis spectroscopy.

2.3.4. Direct MALDI-MS identification of captured carbohydrate on magnetic beads

Mass spectrometry plays a dominant role in profiling complex carbohydrate structures in a sensitive and accurate manner [18]. Direct mass-spectrometric detection of carbohydrates and glycosylated peptides and proteins from complex sources such as blood serum/plasma or urine is more expected for efficiency but still quite difficult due to highly abundant proteins interfering with the mass spectrometric analysis. Therefore, enrichment and purification of the target carbohydrates and glycoconjugates are essential prior mass spectrometric analysis. Streptavidin-biotin/magnetic beads technique has been widely used for rapid biomolecules separation [19]. The use of biotin boropolymer combined with magnetic beads-assisted separation and subsequent MALDI mass

spectrometry ideally fulfills carbohydrates and glycoconjugates identifying requirements. To test this, first, biotin boropolymer **6** was immobilized onto the surface of streptavidin-derivatized magnetic beads (Invitrogen) by incubating **6** with the magnetic beads in PBS (pH 7.4) buffer at room temperature, followed by thoroughly washing with PBS (pH 7.4) buffer. Next, glyco-capturing was conducted by incubating boropolymer-functionalized magnetic beads with lactose used as model carbohydrate in PBS (pH 8.3) buffer at room temperature, followed by thoroughly washing with PBS (pH 8.3) buffer. Finally, direct determination of captured lactose on the magnetic beads was conducted by spotting lactose-captured magnetic beads onto a MALDI sample plate with matrix of 2,5-DHB in Acetonitrile-0.1% TFA (2:1) and subsequent MALDI mass analysis. As shown in Figure 5, successful capturing and identification of the model carbohydrate lactose, which showed as 365.2 [M+Na] and 381.1 [M+K] positive cations, was confirmed with biotin boropolymer **6** immobilized onto streptavidin-magnetic beads (Figure 5A), whereas there is no lactose capturing confirmed by using streptavidin-magnetic beads but in the absence of biotin-boropolymer **6** (Figure 5B). Furthermore, this technique has been confirmed with other monosaccharide such as galactose. The glyco-affinity capturing selectivity of the present system is under investigating and will be reported accordingly. This result demonstrated the concept of efficient glyco-affinity capturing and direct MALDI-MS identification by immobilizing biotin boropolymer onto magnetic bead surface through specific streptavidin-biotin binding.

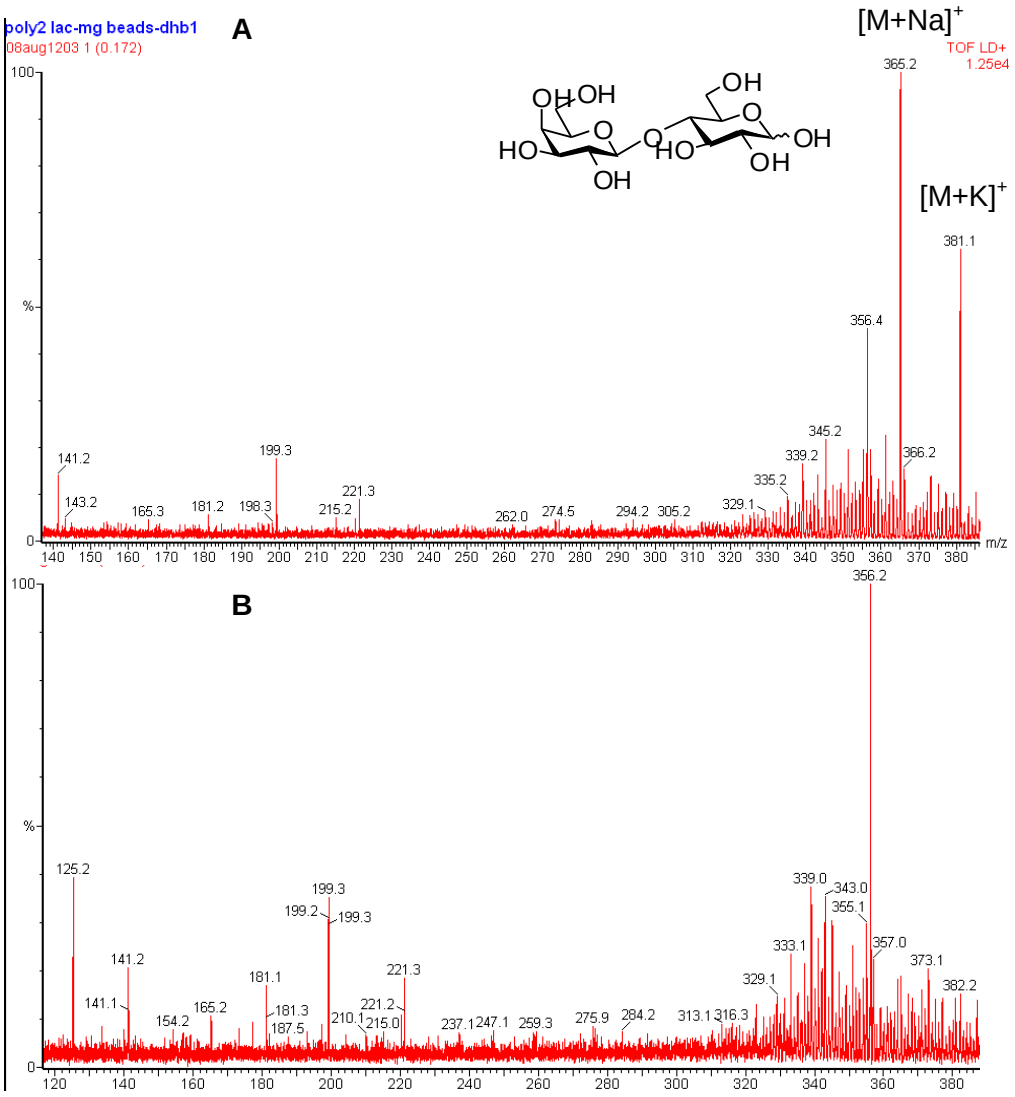


Fig. 5 Direct MALDI-MS (Matrix of 2,5-DHB (6 mg/mL) in Acetonitrile-0.1% TFA (2:1)) identification of captured lactose: Magnetic beads with biotin boropolymer **6** (A), magnetic beads without biotin boropolymer **6** (B).

2.4. Conclusion

A biotin chain-end functionalized boropolymer was synthesized *via* arylamine initiated cyanoxyl-mediated free radical polymerization as a functional glyco-affinity macroligand. The specific streptavidin binding capacity of biotin boropolymer was confirmed by streptavidin-HABA assay, while the specific carbohydrate binding capacity of biotin boropolymer was evaluated by Alizarin Red S binding assay. A strategy for oriented immobilization of boropolymer onto magnetic bead surface through specific streptavidin-biotin binding and its glyco-affinity capturing and direct MALDI-MS identification were demonstrated. It is a unique advantage to use magnetic beads and chain-end functionalized boropolymer as a glyco-affinity macroligand for efficient purification or enrichment of carbohydrates and glycocojugates. By optimizing the polymer and phenylboronic acid structure features, a specific glyco-capturing system will be obtained. The present approach will greatly enhance the throughput identification of carbohydrates and glycoconjugates known as glycomics and glycoproteomics as well as medical diagnostic applications for carbohydrate biomarkers.

2.5. References

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CHAPTER III

SYNTHESIS AND GLYCO-CAPTURING OF ORIENTED GLYCO-AFFINITY MACROLIGAND

3.1 Introduction

The binding of a variety of boronic acid derivatives with their specific carbohydrates have been explored for fabricating carbohydrate and glycoprotein sensors by immobilization of the boronic acid derivatives onto metallic particles or electrodes. For example, coupling 3-aminophenylboronic acid with dithiodialiphatic acids on either Au-colloids or electrodes,[1] preparation of the phenylboronates-oxyrane mixed functional monolayer,[2] and aminophenylboronic acid monolayer on Au-electrode[3] have been reported for electrochemical sensing of carbohydrates. In addition, boronic acid-containing polymers have been developed as a multivalent carbohydrate binder for enhanced substrate affinity.[4] In our study, we designed a chain-end functionalized boronic acid containing polymer (boropolymer) for oriented glyco-affinity macroligand formation (Figure 1). Chain-end functionalized polymer have been explored for bioconjugation, as they allow for site-specific attachment.[5] Particularly, chain-end functionalized boropolymer facilitates site-specific immobilization required for generating uniformly oriented multivalent carbohydrate binders. It is known that natural glycans are presented as a multivalent ligands; a phenomenon referred to as the "cluster glycoside effect".[6] Therefore, this oriented glyco-capturing macroligand is expected to

provide a three dimensional expression of carbohydrate receptors and thus facilitate enhanced selectivity and affinity for carbohydrates capturing.

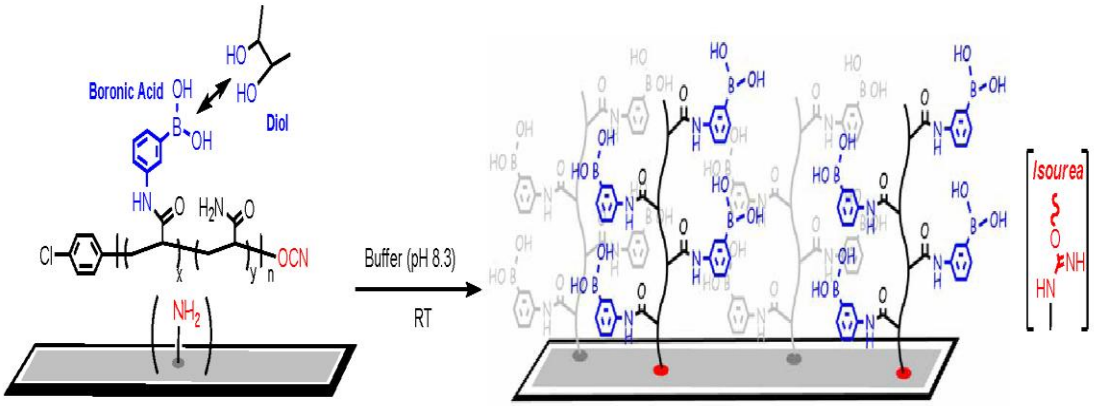
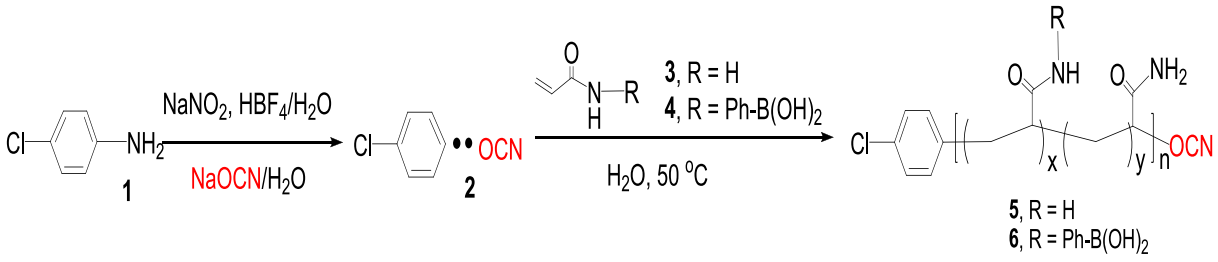


Figure 1. *O*-Cyanate chain-end functionalized boropolymer and its oriented immobilization onto amine surface *via* isourea bond formation as 3D carbohydrate receptor expression

The desire to generate polymers with the functional group at the polymer chain end and with affinity ligand units pending on the polymer backbone poses great challenges, including a requirement for serial protection-deprotection steps or further polymer derivatization after polymerization. For example, reversible addition-fragmentation chain transfer (RAFT) polymerization has been mostly used for the synthesis of chain end functionalized polymers, which is in four steps including protection/deprotection, chain end conversion and further polymer derivatization steps.[7] In our previous studies, we have demonstrated arylamine initiated cyanoxyl-mediated free radical polymerization

(CFRP) as a straightforward approach to synthesize glycopolymers.[8] Notably, this synthetic approach can be performed under aqueous condition and tolerant of a wide range of monomer functionalities, including -OH, -COOH, $-NH_2$ and $-OSO_3^-$ groups and thus does not need protection and deprotection steps. In the present study, we examined another attractive feature of this CFRP providing a polymer with *O*-cyanate group at the polymer chain-end *in situ*, which might be used for polymer conjugation with amine containing biomolecules *via* isourea bond formation.[9] Furthermore, this CFRP affords a polymer with a phenyl group at the chain end that can be used for the calculation of polymer component content and length by NMR spectroscopy. In the present study, CFRP of acrylated phenylboronic acid by using arylamine as initiator was investigated for preparing *O*-cyanate chain-end functionalized boropolymer (Scheme 1). Specifically, cyanoxyl radicals were generated by an electron-transfer reaction between cyanate anions from a sodium cyanate aqueous solution and aryl-diazonium salts prepared *in situ* through a diazotization reaction of arylamine in water. In addition to cyanoxyl persistent radicals, aryl-type active radicals were simultaneously produced, and only the latter species was capable of initiating chain growth.



Scheme 1. Synthesis of *O*-cyanate chain-end functionalized boronic acid containing polymer (boropolymer) *via* CFRP

3.2. Experimental Section

3.2.1. Materials

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all polymerization reactions.

3.2.2 Method

Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 μm thicknesses on which spots were visualized with UV light or by charring the plate after dipping in 10% H2SO4 in methanol. 1H NMR spectra were recorded with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard. A network/spectrum/ impedance analyzer (Agilent 4395A) was used to measure the frequency and series damping resistance of the QCM simultaneously. AFM imaging experiments were performed by using PicoPlus 3000 (Molecular Imaging, USA).

3.2.3. Synthesis of α O-cyanate boropolymer via cyanoxyl-mediated free-radical polymerization

In a three-neck flask, 13 mg of 4-chloroaniline (0.1 mmol) was reacted with 38 mg of HBF4 (48 wt % aqueous solution, 0.12 mol) in 0.5 mL of water and 0.5 mL of THF at 0 °C under an Ar atmosphere. Adding 8.2 mg of sodium nitrite (0.12 mmol) to the reaction medium then generated the diazonium salt. After 30 min, a degassed mixture of 125 mg (1.76 mmol) of acrylamide, 46 mg (0.24 mmol) of 3-acrylaaminophenyl boronic acid **4**, and 32 mg (0.5 mmol) of NaOCN dissolved in 0.5 mL of water and 0.5 ml of THF were

introduced into the flask containing the diazonium salt above. The polymerization solution was then heated to 60 °C for 16 hrs to yield the co-polymer **6** and dialysis against *di* water for 2 days at room temperature to remove inorganic salt and impurities. A series of chain-end functionalized boropolymers of varying molecular weight were prepared by altering the ratio of monomer to initiator concentrations. The conversion yield was about 50-70%, which was determined by weight for the resultant boropolymers.

3.2.4. Synthesis of α O-cyanate polyacrylamide via cyanoxyl-mediated free-radical polymerization

In a three-neck flask, 13 mg of 4-chloro aniline (0.1 mmol) was reacted with 38 mg of HBF4 (48 wt % aqueous solution, 0.12 mol) in 0.5 mL of water and 0.5 mL of THF at 0 °C and under an Ar atmosphere. Adding 8.2 mg of sodium nitrite (0.12 mmol) to the reaction medium then generated the diazonium salt. After 30 min, a degassed mixture of 159 mg (2.24 mmol) of acrylamide and 32 mg (0.5 mmol) of NaOCN dissolved in 0.5 mL of water and 0.5 mL of THF were introduced into the flask containing the diazonium salt. The polymerization solution was then heated to 60 °C for 16 hrs to yield the polyacrylamide and dialysis against *di* water for 2 days at room temperature to remove inorganic salt and impurities.

3.2.5. Synthesis of C-13 labeled α O-cyanate boropolymers via cyanoxyl-mediated free-radical polymerization

In a three-neck flask, 13 mg of 4-chloro aniline (0.1 mmol) was reacted with 38 mg of HBF4 (48 wt % aqueous solution, 0.12 mol) in 0.5 mL of water and 0.5 mL of THF at 0 °C and under an Ar atmosphere. Adding 8.2 mg of sodium nitrite (0.12 mmol) to the reaction medium then generated the diazonium salt. After 30 min, a degassed mixture of 125 mg (1.76 mmol) of acrylamide, 46 mg (0.24 mmol) of 3-acrylaaminophenyl boronic acid **4**, and 41 mg (0.5 mmol) of C-13 labeled KOCN (purchased from Icon Services) dissolved in 0.5 mL of water and 0.5 ml of THF were introduced into the flask containing the diazonium salt. The polymerization solution was then heated to 60 °C for 16 hrs to yield the co-polymer **7** and dialysis against *di* water for 2 days at room temperature to remove inorganic salt and impurities.

3.2.6. Conversion of cyanate (OCN) boropolymer 6 to a hydroxyl end boropolymer 8

Pyridine (0.5 mL) was added to a solution of boropolymer **6** (20 mg) in water (2 mL). The mixture was stirred at room temperature for 8 hrs, followed by dialysis against *di* water at room temperature for 2 days to remove excess pyridine and product glutaconic aldehyde.

3.2.7. Conversion of C-13 labeled cyanate (OCN) boropolymer 7 to a hydroxyl end boropolymer 9

Pyridine (0.5 mL) was added to a solution of C-13 labeled boropolymer **7** (20 mg) in water (2 mL). The mixture was stirred at room temperature for 8 hrs, followed by dialysis

against di water at room temperature for 2 days to remove excess pyridine and product glutaconic aldehyde.

3.2.8. Oriented immobilization of boropolymer onto Au-electrode

Oriented immobilization of boropolymer on Au - electrode was done by coupling the cyanate functionalized boropolymer to amine terminated SAM modified Au electrode. Prior to the modification process, the Au coated quartz crystal was cleaned by sequential immersion in a concentrated nitric and sulfuric acid mixture, biological grade water and ethanol in series for three times to remove impurities, and then dried with nitrogen. After the complete cleaning, amine-active self-assembled monolayer was formed by immersing one side of the Au coated quartz crystal or Au-plate in 10 mg/mL cysteamine solution in ethanol at 4 oC for over night. The Au electrode surface was then rinsed with ethanol and biograde water to remove the weakly adsorbed cysteamine. Finally 20 µL 4 mg/mL boropolymer was added to the cysteamine modified surface for additional 10-12 hrs. This immobilized procedure was monitored using a network/spectrum/ impedance analyzer (Agilent 4395A).

3.2.9. Detection of glycoprotein (Lac-BSA) binding with boropolymer immobilized onto Au-electrode

The frequency changes were monitored over time using a network/spectrum/ impedance analyzer (Agilent 4395A) in the presence of increasing concentrations of galsctose (from 0.2 mg/mL to 7.6 mg/mL), lactose (from 0.2 mg/mL to 7.6 mg/mL), and BSA-Lactose

copnjugate (purchased from Sigma, from 5 μg/mL to 195 μg/mL) in Bi-carbonate buffer (pH 8.3) at room temperature.

3.2.10. Amine modification of mica surface with 3-aminopropyl triethoxysilane (APTES)

The freshly cleaned mica substrates were prepared. Briefly, freshly cleaved mica surfaces were immersed into an aqueous solution of 1% (v/v) 3-aminopropyl triethoxysilane (APTES, purchased from Sigma) for a few minutes. Then the mica substrates were carefully rinsed with water and blown dry by argon for preparing the sample.

3.2.11. Immobilization of polymer(s) on amine modified mica surface

A stock solution (0.2 mg/mL) of either the boro-polymer **6** or the control polymer **5** is prepared in Bi-carbonate (pH 8.3) buffer (0.1 M NaHCO3 and 0.5 M NaCl). 10 μL of the stock solution was added to 5 mL of the bicarbonate buffer and the above-modified mica substrates were incubated in 5 mL of the polymer(s) solution and left for overnight at 4 ^{o}C. The specimens were then thoroughly washed with the buffer and sonicated to remove any non-specific binding. The mica substrates were then rinsed with water and dried immediately with argon for AFM imaging study.

3.2.12. Capture of lactose-BSA & BSA onto boropolymer modified mica surface

A stock solution (0.2 mg/mL) of either the BSA (Bovine serum albumin) or the Lactosyl-BSA (Bovine serum albumin) is prepared in Bi-carbonate buffer (0.1 M NaHCO3 and 0.5 M NaCl). From the above stock solutions 0.1 μg/mL solutions were prepared and Boro-

polymer modified mica substrates were incubated in 10 mL of those diluted solutions and incubated for about 4 hrs at 4 ^{o}C. The specimens were then thoroughly washed with the buffer and sonicated to remove any non-specific binding. The mica substrates were then rinsed with water and dried immediately with argon for AFM imaging study.

3.2.13. Capture of lactose-BSA on to control polymer modified mica surface

A stock solution (0.2 mg/mL) Lactose-BSA is prepared in Bi-carbonate (pH 8.3) buffer (0.1 M NaHCO3 and 0.5 M NaCl). From the above stock solutions 0.1 μg/mL solutions were prepared and polymer(s) (control & boropolymer) modified mica substrates were incubated in 10 mL of those diluted solutions and incubated for about 4 hrs at 4 ^{o}C. The specimens were then thoroughly washed with the buffer and sonicated to remove any non-specific binding. The mica substrates were then rinsed with water and dried immediately with argon for AFM imaging study.

3.2.14. Capture of lactosyl-BSA on to the boropolymer(s) modified mica surface saturated with lactose

A stock solution (0.2 mg/mL) of Lactose-BSA is prepared in Bi-carbonate buffer (0.1 M NaHCO3 and 0.5 M NaCl). From the above stock solutions 0.1 μg/mL solution is prepared. Lactose solution (1 mg/mL) is also prepared in Bi-carbonate buffer (0.1 M NaHCO3 and 0.5 M NaCl). Boropolymer modified mica substrate is incubated in 10 mL of the lactose solution for about 4 hrs at 4 ^{o}C. The specimens were then thoroughly washed with the buffer and sonicated to remove the unbound lactose. The mica substrates were then rinsed with water and dried. After the saturation with the lactose the mica

substrate is incubated in 10ml the above-diluted solution of the Lactose-BSA for about 4 hrs at 4 oC. The specimens were then thoroughly washed with the buffer and sonicated to remove any non-specific binding. The mica substrates were then rinsed with water and dried immediately with argon for AFM imaging.

3.3. Results and Discussion

3.3.1. Characterization of OCN chain-end functionalized polymer by ^{1}H NMR spectroscopy

The resultant boropolymers were characterized by ^{1}H-NMR spectroscopy. It is noteworthy that the phenyl group at the end of the boropolymer provides a convenient mechanism for calculating boronic acid content and the molar mass through comparison of integrated phenyl proton peaks with those of backbone protons. As shown in Figure 2, comparison of the integrated signal from the phenyl protons (7.00 – 7.80 ppm) with that due to the polymer backbone methine (2.20 ppm) and methylene (1.60 ppm) indicated an average polymer composition of 5 phenylboronic acid and 70 acrylamide units for boropolymer **6** (Figure 2B). As shown in the control polymer **5** without phenylboronic acid (Figure 2A), the phenyl protons (6.81 ppm, 2H and 7.60 ppm, 2H) were clearly assigned and used for calculation of the polymer composition *via* proton integrations.

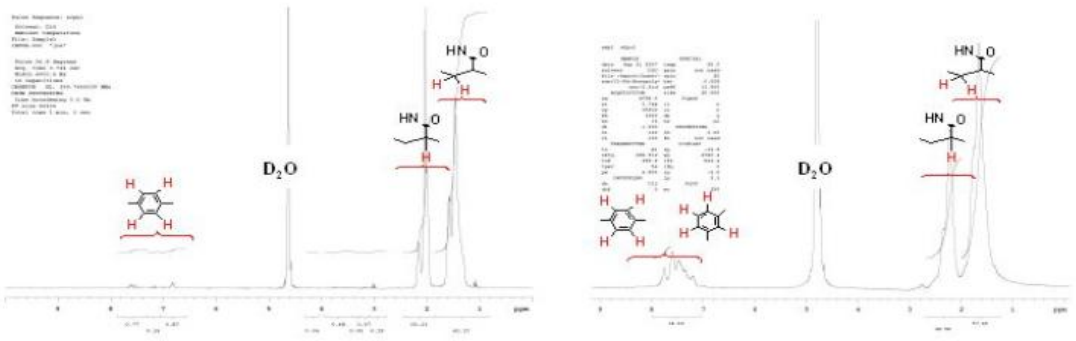


Figure 2. ^{1}H NMR spectra of OCN chain-end functionalized polymer in D_2O

3.3.2. Characterization of OCN and OH chain-end functionalized boropolymers by ^{13}C NMR spectroscopy

^{13}C NMR was used to confirm the chain end OCN group by synthesizing ^{13}C labeled OCN chain end functionalized boropolymer (7) *via* the same CFRP by using KO^{13}CN as initiator. As shown in Figure 3, a sharp ^{13}C signal at 134 ppm was observed for ^{13}C labeled OCN chain end in boropoymer 7, while it appears as a week signal for non-labeled OCN chain end boropolymer 6 since there is only one OCN group in each polymer. Furthermore, the *O*-cyanate group can be converted to a hydroxyl group by treatment with pyridine in water.[10] As shown in Figure 3C, sharp ^{13}C signal at 134 ppm disappeared in boropolymer 9 with OH at the chain end, which was obtained from ^{13}C labeled OCN chain end functionalized boropoymer 7 when treated with pyridine in water. Similarly, signal at 134 ppm disappeared in boropolymer 8 with OH at the chain end, which was obtained from OCN chain end functionalized boropoymer 7 when treated with

pyridine in water. These results confirmed the existing of the OCN group on the chain end of boropolymer.

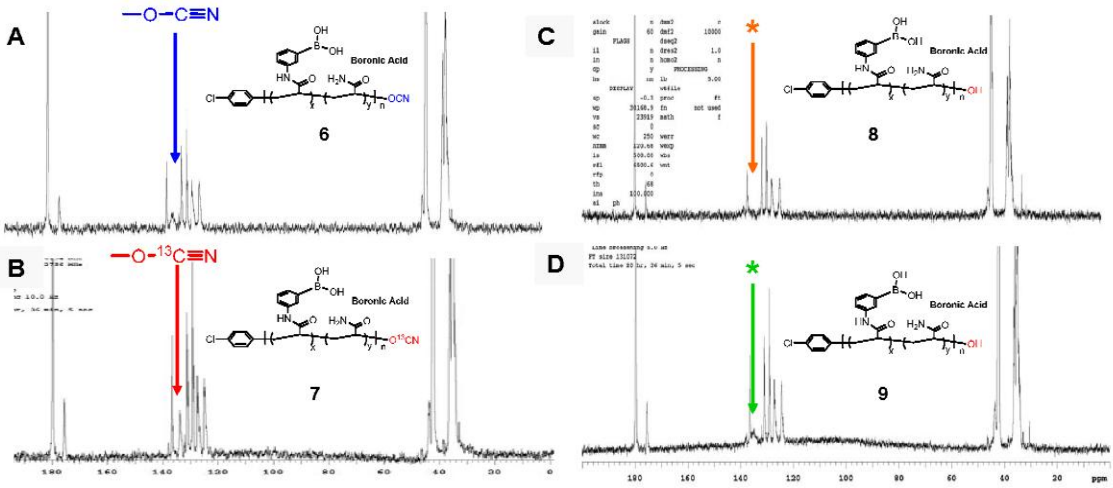


Figure 3. ^{13}C NMR spectra of OCN and OH chain-end functionalized boropolymers in D_2O

3.3.3. Boronic acid-sugar specific binding assay of boropolymer

The ability of the boropolymer to specifically bind carbohydrate was accessed by Alizarin Red S. (ARS)-binding assay.[11] ARS displays a dramatic change in fluorescence intensity and color in response to the binding of a boronic acid and has been used as a general reporter for studying carbohydrate–boronic acid binding interactions. In this study, the effect of boropolymer (BP) on the spectroscopic property of ARS in aqueous solution was examined with UV-visible spectrometer. As shown in Figure 4, ARS showed a color change from deep red (Figure 4, trial A) to yellow (Figure 4, trial A + B) in the presence of BP in aqueous solution at pH 7.4. Galactose was used as a model compound to determine the binding between BP and carbohydrates in a three-component system. As a result, the galactose–PB complex formed immediately upon adding

galactose into the solution of PB with ARS, a corresponding change in solution color from yellow (Figure 4, trial A + B) to red (Figure 4, trial A + B + C). This result demonstrated the specific carbohydrate binding capacity of the boropolymer.

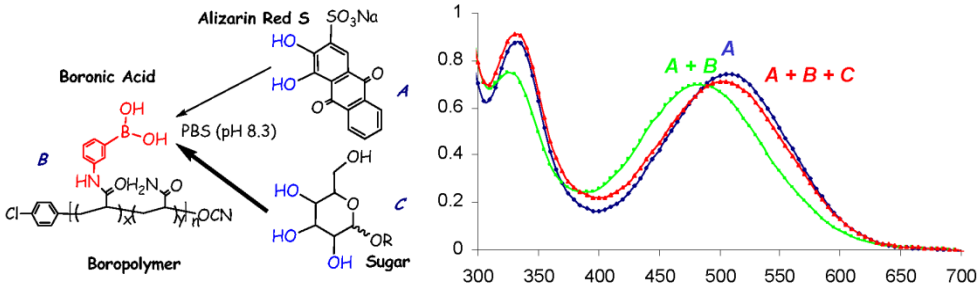


Figure 4. Boronic acid-sugar specific binding assay of boropolymer (6) monitored by UV-vis spectroscopy: A = Alizarin Red S, A + B = Alizarin Red S + boropolymer, A + B + C = Alizarin Red S + boropolymer + galactose

3.3.4. Characterization of immobilised boropolymer on amine surface by Cyclic Voltammetry and QCM

Cyanate-amine conjugation to form isourea bond has been widely used for biomolecule conjugation.[9] In the present study, the *O*-cyanate-based oriented immobilization of boropolymer onto amine surface was validated by using QCM technique. QCM allows the monitoring of both the dynamics of the polymer immobilization processes and the response behavior of carbohydrate binding with immobilized boropolymer on the Au surface of the QCM sensor sensitively and also in real time. Initially, an amine functionalized Au surface was prepared by coupling cysteamine onto the Au sensor chip *via* thiol-Au chemistry. The resulting Au sensor chip with terminal amino group was then

coupled with *O*-cyanate chain-end functionalized boropolymer **6** in bicarbonate buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) to form an oriented boropolymer surface through isourea bond formation. The real time frequency response of QCM upon immobilization of boropolymer onto the amine functionalized sensor surface was shown in Figure 5. An approximate 290 Hz frequency decrease was observed before and after washing confirming the successful immobilization of the *O*-cyanate chain-end functionalized boropolymer on the cysteamine modified Au electrode surface.

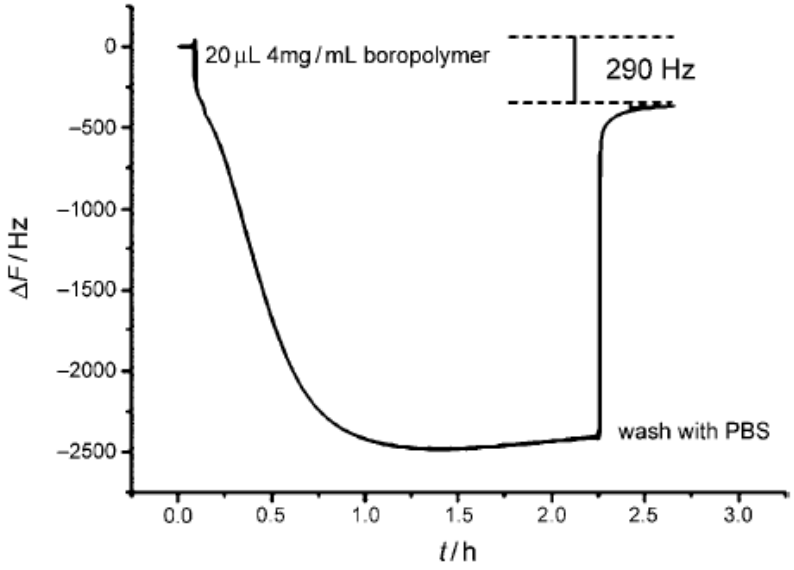


Figure 5. CVs plots for bare Au, cysteamine-Au, boropolymer-cysteamine-Au modified electrode in 5 mM $Fe(CN)_6^{3-/4-}$/0.1 M KCl solution (A), Scan rate at 50mV/s (A) and frequency change vs time curves upon immobilization of boropolymer onto QCM senor surface (B).

3.3.5. Carbohydrate and glyco-conjugate capturing of immobilized boropolymer by Quartz Crystal Microbalance

The QCM responses caused by the interaction between the immobilized boropolymer on the sensor surface and analyte carbohydrates and glycocojugates were evaluated by measurement of frequency change. In present study, galactose, lactose and lactose-modified BSA were used as model carbohydrate and glycoconjugate. It was found that the frequency decrease is about 20 Hz when adding 200 mg/mL of galactose (Figure 6A), while 25 Hz decreased when adding 200 mg/mL of lactose (Figure 6B) to the boropolymer sensor. Particularly, similar frequency decrease needs only 200 mg/mL of lactose-modified BSA (Figure 6C). It can be explained that the QCM will give different sensitivity depending on the mass of target carbohydrates and glycoconjugates. These results confirmed that carbohydrate binding was mainly attributed to the carbohydrate interaction with the boronic acid groups of the boropolymer. Under optimized experimental conditions, quantification of lactose-modified BSA using the developed sensor was carried out for a concentration range of 0.5 to 200 μg/mL (Figure. 6D). A good linear relationship response to lactose-modified BSA concentration within the range from was obtained with a corelation coefficient of 9.821×10^{-1}. The detection limit of this sensor was 0.5 μg /mL. The high sensitivity and low detection limit of this proposed sensor could be attributed to the multivalent binding between the boropolymer and lactose-modified BSA. The glyco-affinity capturing selectivity correlated to the boropolymer structure and density of immobilized boropolymer of the present system is under investigating and will be reported accordingly.

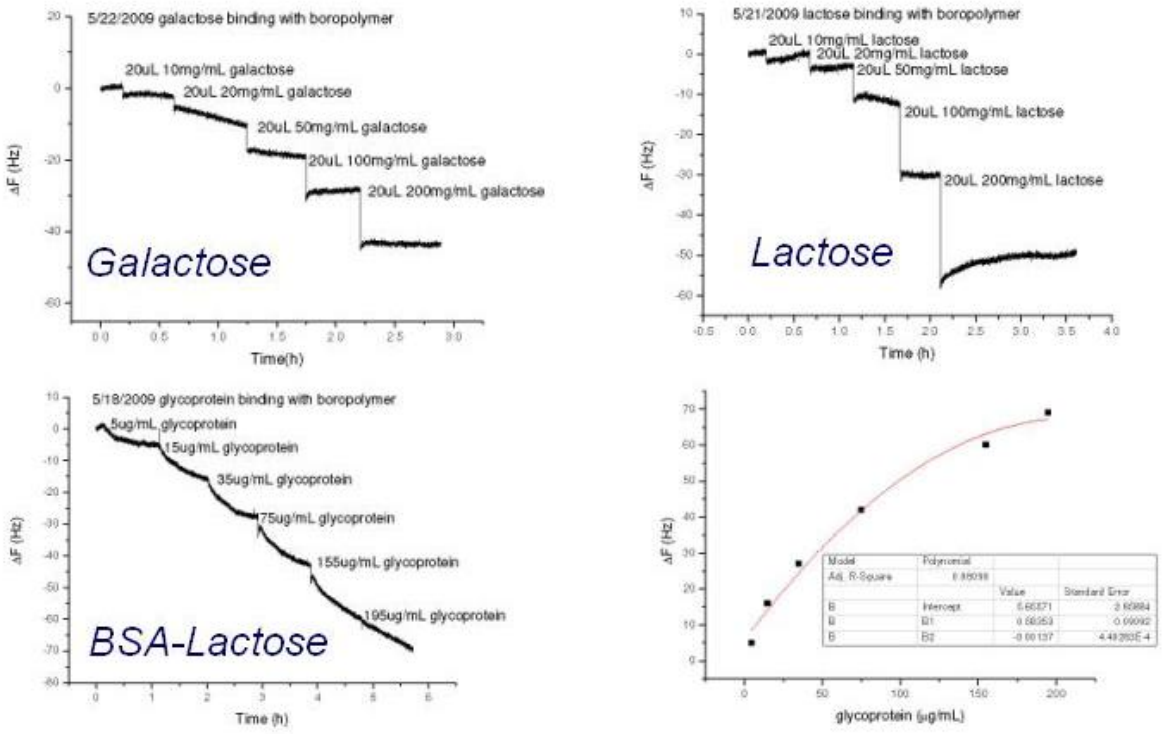


Figure 6. Frequency changes vs time curves of carbohydrate and glyco-conjugate capturing onto immobilized boropolymer QCM sensor surface

3.3.6. Monitoring of boropolymer immobilization and glyco-protein capturing onto immobilized boropolymer surface using AFM

The immobilization of *O*-cyanate chain-end functionalized boropolymer onto amine surface and its binding capacity with glycoconjugate were further confirmed by Atomic Force Microscopy (AFM). As shown in Figure 7, the immobilized boropolymer onto amine-modified mica was observed after immobilization reaction in bicarbonate buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) at room temperature for 12 hrs followed with washing with PBS (pH 7.4) buffer (Figure 7B), while there was no immobilized boropolymer observed for mica surface without amine groups (Figure 7A). The immobilized polymer molecules appear as dots corresponding to the pancake-like

collapsed conformation that is commonly observed with flexible polymers grafted on a surface.[12] Next, glycoconjugate binding was conducted by incubating boropolymer-anchored mica in the presence of lactose–BSA conjugate in PBS (pH 8.3) buffer at room temperature for 2 hrs followed by washing with PBS (pH 8.3) buffer three time to remove unbound lactose-modified BSA. The specific binding of lactose–BSA conjugate was observed on boropolymer-modified mica (Figure 7D), while no lactose–BSA conjugate binding was observed when un-modified mica were used as control (Figure 7C). Furthermore, no apparent lactose–BSA conjugate binding were observed on free lactose pre-treated boropolymer-modified mica (Figure 7E) and on homopolymer of polyacrylamide **5** (without boronic acid) immobilized onto mica surface (Figure 7F). The glycoconjugate-bounded immobilized polymer molecules appear as "tooth" shape aggregate with average size of 80 nm wide x 14 nm high, which is different from the pancake-like collapsed conformation of the immobilized polymers above. These AFM results further confirmed the successful oriented immobilization of boropolymer and its specific glycoconjugate capturing capacity.

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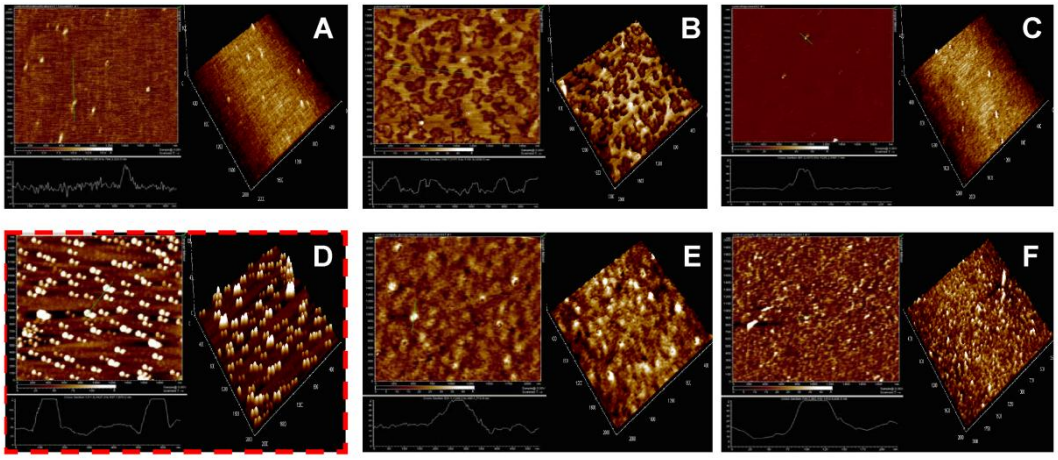

Figure 7. AFM Monitoring of boropolymer immobilization and glyco-protein capturing onto immobilized boropolymer surface: A: mica surface without amine groups; B: immobilized boropolymer (6) on Mica surface with amine; C: Lactosylated BSA + Mica/PBS (pH 8.3); D: Lactosylated BSA + Boropolymer-Mica/PBS (pH 8.3); E, Lactosylated BSA + Free Lactose + Boropolymer-Mica/PBS (pH 8.3); F: Lactosylated BSA + polymer (5)-Mica/PBS (pH 8.3).

3.4. Conclusions

An *O*-cyanate chain end functionalized boronic acids containing polymer was synthesized *via* arylamine initiated cyanoxyl-mediated free-radical polymerization in one-pot fashion. The synthesis is straightforward without protection/deprotection and subsequent conversion needed. A strategy for oriented boropolymers immobilization onto amine surface through isourea bond formation and its carbohydrate and glycoconjugate capturing capacity were confirmed by both QCM and AFM techniques. The immobilization was performed in mild aqueous condition. The resultant 3D carbohydrate receptor will find important application for efficient carbohydrate sensing and capturing with enhanced selectivity and affinity. On

the other hand, because amine-grafted surface can be assembled on various supports such as gold, glass, silica, or alumina, the presented oriented multivalent carbohydrate receptor could be extended to many support materials. Therefore, the oriented glyco-capturing macroligand can be used for efficient carbohydrate and glycoconjugate purification and identification and thus is expected to constitute a core strategy of glycomics and glycoproteomics and carbohydrate sensing applications.

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CHAPTER IV

MULTIVALENT INTERACTION BASED CARBOHYDRATE BIOSENSORS FOR SIGNAL AMPLIFICATION

4.1. Introduction

Understanding the role of carbohydrates in various physiological processes and their biological functions requires methods to measure and characterize molecular interactions between carbohydrates and their corresponding receptors. Unlike proteins and DNAs, most of carbohydrates are complex, irregular and have only one kind of functional group (hydroxy) with different stereochemistry, the hydroxylated exteriors of the carbohydrates may blend well with water, making the recognition of different carbohydrates in aqueous solution highly challenging[20]. Additionally, carbohydrates lack of chromophores or flurophores in their structure and their detection by optical methods is very difficult. In the past decades, various indirect and direct methods including electrochemical[9], refractive index[16], mass spectrometry[12], light scattering[37], chiroptical[11], pre- and post column derivatization reactions for optical detection (UV-Vis, fluoresence)[2,14] and post column enzyme reactor[24] etc. were demonstrated. However, many of mentioned methods are complex and require the use of expensive instruments. Recent years, carbohydrate biosensors based on enzymatic catalysis[4,11,34] lectin recognition[21,26], microbial interaction[6,13] have been demonstrated and they show great promise. Nevertheless,

carbohydrate biosensors using enzyme, lectin or microbial recognition motifs have short lifetime due to the lack of stability of the proteins and cells, thus require replenishment.

To address the low stability of those natural recognition motifs above, the development of synthetic molecular receptors in recognition of carbohydrates such as boronic acid[36,40] have gained significant momentum in recent years. Boronic acid, first reported by Lorand and Edwards in 1959, is known to form covalent bonds between its 1,2- or 1,3-diol group and the *cis*-diol group of the carbohydrates under alkaline conditions[23]. It has been demonstrated to selectively and reversibly interact with carbohydrates to form esters in aqueous media[17], which supports their use in the development of sensors for selective and continuous carbohydrate monitoring. A variety of boronic acid-based host compounds have been prepared and evaluated as synthetic carbohydrate-binding receptors by immobilizing them either on metallic particles or electrodes for fabricating carbohydrate and glycoprotein sensors. For example, coupling 3-aminophenylboronic acid with dithiodialiphatic acids on either Au-colloids or electrodes[19], phenylboronates-oxyrane mixed functional monolayer[1] and aminophenylboronic acid monolayer on Au-electrode[38] have been reported for electrochemical sensing of carbohydrates or glycoproteins. However, these methods provide only a two dimensional display of monomeric boronic acids and give rise to the low detection sensitivity.

Furthermore, the interaction of carbohydrates with target proteins is not a simple monomeric binding event, but an oligo- or polymeric binding mechanism in the biological processes. For example, the binding of a trivalent oligosaccharide ligand to its

asialoglycoprotein cell surface receptor occurs with a binding constant of 10^8 M^{-1} even though the binding constant of corresponding monovalent interaction is only 10^3 M^{-125}. We have focused on the development of lable free affinity carbohydrate-based biosensors by using label free transducers such as Quartz Crystal Microbalance (QCM) transducers[30,39]. Interfacial mass changes due to the binding between a ligand (boronic acid derivatives) immobilized on a solid support and the analyte (carbohydrates) can result in changes in the QCM oscillation frequency. However, QCM measures only those materials that are acoustically coupled to the sensor surface. It is binding-based detection rather than proximity-based detection (e.g surface plasmon resonance, SPR) and requires the ligand-analyte complex to be rigid (e.g. strongly attached on the sensor surface). Additionally, QCM is a mass sensor. The higher the molecular weight of the target analytes and the higher density of recognition elements immobilized, the bigger the mass change and the bigger signal. The usual big size of ligands such as lectins plus the small molecular weights of carbohydrates especially monosaccharide makes it difficult to obtain an observable signal by QCM due to the low densities of recognition elements immobilized and the low mass of the analytes. Therefore, exploring the characteristics of the polyvalent interactions of carbohydrate in nature, we designed the boronic acids polymers that are capable of reversible formation of covalent bonds with the diol functionalities of carbohydrates in form of cyclic esters. Moreover, the self-assembled carbohydrates on the Au - nanoparticles (AuNPs) were used to amplify the mass of the carbohydrate analytes as well as to generate a multivalent carbohydrate moiety. Our approach allows both the ligands (boronic acids) and analytes (carbohydrates) to be polyvalent so that the interaction of the carbohydrates and the ligands are simultaneously

amplified multiple times. Specifically, a functional boronic acid-contain polymer (boropolymer) containing multivalent boronic acids (more than four boronic acid groups on one polymer) appending onto polyacrylamides backbone and *O*-cyanate (OCN) chain end group (figure 2A) was synthesized. The OCN group is known to form isourea bond with amine group and thus facilitates an oriented immobilization onto amine functional group decorated surfaces. On the other hand, the challenge of the small molecular weight of saccharides was addressed by the multiple copies of saccharides immobilized on the AuNPs carriers. AuNPs are ideal carriers in our carbohydrates sensor systems for several reasons: first, AuNPs can be easily prepared in a wide range of sizes, from about 2 nm to above 100 nm; second, the specific activities of carbohydrates can be retained when coupling carbohydrates to AuNPs[7]; third, the carbohydrate - AuNPs conjugates can provide amplification of the detection signal due to the large surface/volume ratio. The binding between the polyvalent boropolymers and carbohydrate - AuNP conjugates was characterized by the QCM, Ultraviolet-visible Spectroscopy (UV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

4.2. Experimental Section

4.2.1. Materials

Cysteamine, Bovine serum albumin (BSA) and Sodium citrate dihydrate were purchased from Aldrich–Sigma Co. Hydrogen tetrachloroaurate trihydrate was purchased from Acros Organics. PEG-thiol was obtained from Nektar. The AuNPs (mean size: 15nm, 30nm, 50nm) were prepared via citrate reduction of hydrogen tetrachloroaurate according to the literature[22]. Au nanorods (0.18nm) were prepared via seed-mediated growth

procedure according to the literature[5]. Bi-carbonate buffer (pH 8.3) was prepared using 0.1M $NaHCO_3$ and 0.5M NaCl, then adjust the pH to 8.3. Unless otherwise indicated, all chemicals, reagents and solvents were obtained from Sigma and used as supplied without further purification.

4.2.2. Preparation of AuNP-carbohydrate conjugates

Five kinds of thiolated carbohydrate derivatives, namely mannose, galactose, glucose, fucose and maltose thiols were synthesized. The preparation, purification and characterization of all the functionalized carbohydrate thiols are described in details in the supporting information. The structures of these five carbohydrate thiols consist of three components. The first is the carbohydrates linked through their respective reducing end; the second is the poly(ethylene glycol) (PEG; $[OCH_2CH_2]_nOH$, n=3) chain; and the third component is the alkyl portion terminated with a thiol group, which can adsorb strongly on the Au surface via Au-S bond. AuNP - carbohydrate conjugates was made by coupling each carbohydrate thiol to the AuNPs via self-assemble process. 0.5 mg of those synthesized carbohydrates were dissolved in the 0.1 mL AuNPs (15 nm, 30 nm, 50 nm) and Au-nanorods (0.18 nm) solution separately, then stand for 24 h with thorough mixing to allow self-assembly of carbohydrate thiolates on the AuNPs surfaces. The AuNPS - carbohydrate conjugates were evaluated using UV - spectroscope.

4.2.3. Synthesis of boronic acid - containing polymer

The boropolymer was prepared through arylamine initiated cyanoxyl-mediated free radical polymerization, which has been previously demonstrated as a straightforward approach to synthesize chain end functionalized polymers in our laboratory[15,32,33]. Briefly, the boropolymer was synthesized by a copolymerization between acrylated phenylboronic acid and acrylamide initiated by cyanoxyl radicals, which was generated by reaction between cyanate anions from a sodium cyanate aqueous solution and aryl - diazonium salts prepared *in situ* through a diazotization reaction of arylamine in water. Detail synthesis has been described in another paper[31]. Structure of boropolymer is shown in figure 2A.

4.2.4. Oriented immobilization of boropolymer

Oriented immobilization of boropolymer on Au - electrode was done by coupling the cyanate functionalized boropolymer to amine terminated SAM modified Au electrode. Nonpolished AT-cut Au quartz crystals (10-MHz, area 0.23 cm^2) were mounted in a custom-made Kel-F cell sealed with two Viton O-rings. Prior to the modification process, the Au coated quartz crystal was cleaned by sequential immersion in a concentrated nitric and sulfuric acid mixture, biological grade water and ethanol in series for three times to remove impurities, and then dried with nitrogen. After the complete cleaning, amine-active self-assembled monolayer was formed by immersing one side of the Au coated quartz crystal or Au-plate in 10mg/mL cysteamine solution in ethanol at 4℃ for overnight. The Au electrode surface was then rinsed with ethanol and biograde water to remove the weakly adsorbed cysteamine. Finally 20 μL 4mg/mL boropolymer was added

to the cysteamine modified surface for additional 10-12 hours. The coupling between amine and cyanatefunctional groups of boropolymer will allow oriented immobilization of boropolymer on the cysteamine modified electrode as shown in scheme 1.

4.2.5. UV-spectroscope

UV-visible absorption measurements were performed using a Cary 100 Bio UV-visible spectrophotometer at room temperature. Quartz cuvette with an optical light path of 1mm was used to contain the sample while collecting the spectra in the wave length range from 400 to 800 nm.

4.2.6. QCM measurement

The boropolymer modified Au quartz electrode was mounted in the Kel - F cell, and the QCM cell filled with 1 mL of Bi - carbonate buffer (pH 8.3) was placed in a Faraday cage at room temperature. Agilent 4395A was used to measure the frequency of the QCM simultaneously. Contents of the QCM cell were continuously stirred before, during, and after the addition of analyte, which was added to the cell in certain volumes of carbohydrates - AuNPs conjugates. The relationship between the change in resonant frequency (ΔF) resulting from a change in mass (Δm), was given by the Sauerbrey's equation, $\Delta F = -2\Delta m n F_0^2 (\rho_q \mu_q)^{-1/2} A^{-1}$. Where n is the overtone number, μ_q is the shear modulus of the quartz (2.947×10^{11} g cm^{-1} s^{-2}), and ρ_q is the density of the quartz (2.648 g cm^{-3}). According to the Sauerbrey's equation, our fitted frequency change of 1 Hz corresponds to a mass increase of 1 ng for the 10 MHz quartz crystal used in this work.

4.2.7. Electrochemical characterization

CV and EIS were carried out with a Potentiostat–Galvanostat (EG&G PARC Model 2263). A three - electrode system with a bare or modified Au - plate electrode, a platinum wire counter electrode and a Ag/AgCl reference electrode (with saturated KCl) was employed. All experiments were performed in Kel - F cell filled with 2 ml of 0.1 M KCl and 5 mM $K_3Fe(CN)_6$ /$K_4Fe(CN)_6$ at room temperature. CV was scanned at 50 mv/s. EIS was obtained with ac amplitude of 10 mV, and frequency from 100 kHz to 100 mHz. The bias potential was set at open circuit potential.

4.3. Results and Discussion

4.3.1. Investigation of carbohydrates conjugated AuNPs

Since the carbohydrates were chemically modified with thiol groups that can covalently attach to the Au surface, the carbohydrates can therefore coat on the AuNPs surface via self - assemble process. The carbohydrates were conjugated to the AuNPs as outlined in Figure 1. This process was characterized by UV - vis Spectroscopy based on the unusual dependence of the optical and electronic properties on the AuNPs size and shape[27]. The UV - vis spectrum of 15nm AuNPs shows a plasmon absorption peak at 520 nm. Then this peak was shifted to 526 nm after carbohydrate binding (Figure 1). The 6 nm red - shift in the peak Plasmon absorption is related to a change in the local dielectric constant around the AuNPs as a result of adsorption carbohydrates onto the AuNPs.

The concentration of AuNPs was measured by UV - vis and calculated as 1.9 nM for 15 nm AuNPs, 0.23 nM for 30 nm AuNPs and 0.10 nM for 50 nm AuNPs, according to Beer's law by using known values of the extinction coefficients for AuNPs (4.2×10^8 L

mol^{-1} cm^{-1} at λmax_{520} for 15 nm AuNPs, 3.7 $\times 10^9$ L mol^{-1} cm^{-1} at λ_{528} for 30 nm AuNPs and 1.5 $\times 10^{10}$ L mol^{-1} cm^{-1} at λ_{540} for 50nm AuNPs, respectively)[8,18]. The final concentrations of carbohydrates were 16 mM for fucose derivative, 15mM for mannose, galactose, glucose and 10 mM for lactose and maltose. Thus, we intentionally have carbohydrate concentrations three or four fold higher than that of the AuNP carriers to ensure the each AuNP - carbohydrate conjugate has the maximum surface coverage of carbohydrates on the AuNP surface so that a quantitative analysis of carbohydrate - boropolymer binding can be achieved.

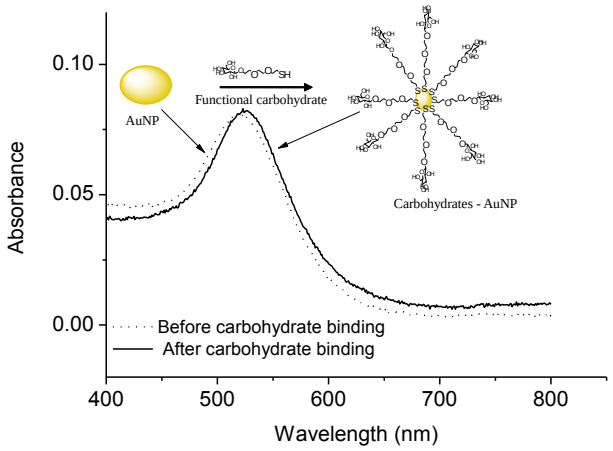

Figure 1. UV-vis spectra of carbohydrate stabilized AuNPs (15nm) solution before (dot line) and after addition of mannose (solid line).

4.3.2. Characterization of boropolymer modified electrode

A SAM of cysteamine was formed on the Au surface of the sensor chip via Au-thiol chemistry. The resulting sensor surface with terminal amino groups was then coupled with a cyanate chain-end functionalized boropolymer to form a SAM of boronic acid. The strong and specific cyanate - amine reaction facilitates high-oriented assembly of boronic acids on the sensor surface[28]. This modification of the Au-electrode is schematically shown in figure 2A. Cyclic Voltammetry Characterizations of the bare Au, cysteamine modified Au, and boropolymer and cysteamine modified Au electrodes were obtained in the presence of 0.5 mM $Fe(CN)_6^{3-/4}$ redox probe as shown in Fig. 2B. Compared with bare Au-electrode, the peak currents reduced after the electrode was modified with cysteamine, indicating that the cysteamine was attached on the Au surface and formed an electron-transfer barrier to the $Fe(CN)_6^{3-/4-}$ redox active couple. Formation of the boropolymer monolayer on Au-electrode led to further decrease of peak currents due to electrostatic repulsion between $Fe(CN)_6^{3-/4-}$ and boropolymer, as boronic acid is in an anionic form in alkaline solution (pH 8.3). This result confirmed the presence of boropolymer on the electrode surface. The same results were also obtained by EIS (Figure 2B, inset).

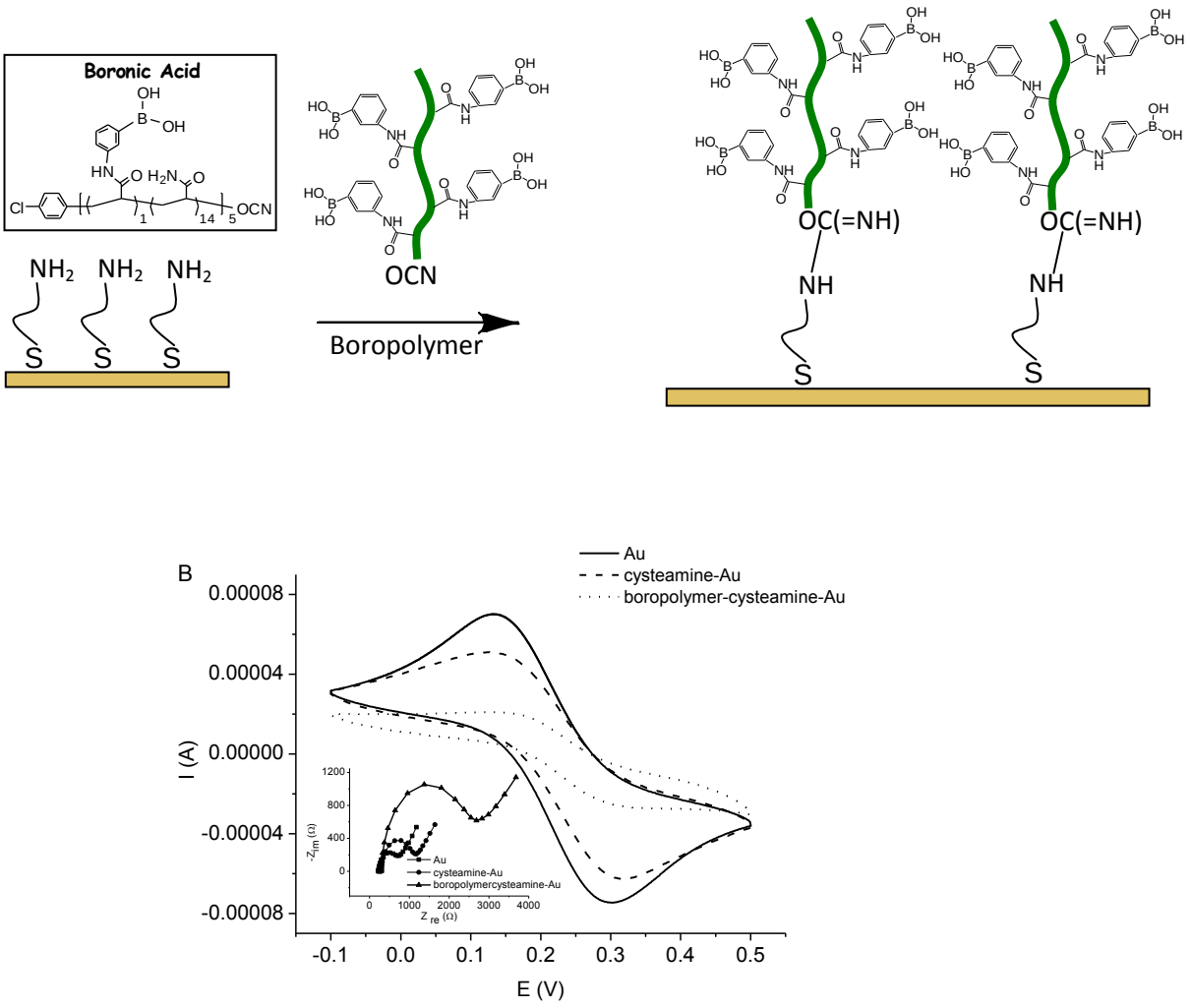

Figure 2. A. Schematic representation of oriented immobilizing boropolymer onto cysteamine -functionalized Au electrode surface. B. CVs and complex impedance (inset) plots for bare Au, cysteamine-Au, boropolymer-cysteamine-Au modified electrode in 5 mM $Fe(CN)_6^{3-/4-}$ / 0.1 M KCl solution. Scan rate, 50 mV/s.

4.3.3. Evaluation of carbohydrate and boropolymer interactions

Monosaccharide is small in size, therefore it is very difficult to obtain high sensitivity using mass sensors such as QCM. As shown in figure 3A, the frequency decrease was only about 7 Hz when 0.1 mg/mL free mannose thiols was added to the boropolymer modified Au sensor surface (curve a, top). However, when treatment of the resulting solution with amplification probe-AuNPs, it yielded a frequency decrease of about 32 Hz (curve a, bottom), indicating that AuNPs would conjugate with functional mannose and the binding of mannose-AuNPs conjugation offered a significant amplification of carbohydrates-boronic acid binding. But addition of AuNPs alone on the boropolymer modified Au sensor surface almost did not give rise to the frequency change (curve c). To evaluate the degree of non-specific interaction between the modified surface and carbohydrate, we performed parallel experiment on the Au surface that was only modified with cysteamine. The frequency response of addition of the 0.1 mg/mL of mannose thiols was found to be 2 Hz (curve b). It confirmed that carbohydrate binding was mainly attributed to the carbohydrates interaction with the boronic acid groups of the boropolymer. These results also revealed that the carbohydrates - AuNPs conjugation amplified the signal of carbohydrate-boropolymer interaction and allowed carbohydrate sensing to be highly specific and sensitive.

To investigate the affinity of the carbohydrate - AuNP conjugates with boropolymer immobilized on the Au sensor surface, five kinds of carbohydrates, mannose, fucose, galactose, glucose, and maltose derivatives conjugated with AuNPs were prepared in the

identical ways. Fig. 3B shows these carbohydrates - AuNPs conjugates binding with boropolymers. As expected, all the boropolymer modified Au quartz crystal electrodes showed significant frequency changes upon the addition of the functionalized carbohydrates - AuNPs conjugates, and the frequency decrease is concentration-dependent. Thus, we will be able to obtain the associate constants (K_a) of the binding between carbohydrates - AuNPs and boropolymer using eq 1. Association constant and dissociation constant (K_d) for the binding between carbohydrates - AuNPs and boropolymer can be evaluated by eq 2[29], where ΔM_{max} is the maximum binding amount, ΔM is the measured binding amount, and [carbohydrates - AuNPs]$_0$ is the original concentration of carbohydrates-AuNPs.

[Boropolymer]+ [carbohydrates-AuNps] $\rightleftharpoons$ [boropolymer-carbohydrates-AuNps] (1)

$$\frac{[\text{carbohydrates-AuNPs}]_0}{\Delta M} = \frac{[\text{carbohydrates-AuNPs}]_0}{\Delta M_{max}} + \frac{1}{\Delta M_{max} K_a} \quad (2)$$

The inset of figure 3B shows the plot of [carbohydrates-AuNPs]$_0$ /ΔM versus [carbohydrates-AuNPs]$_0$ based on the Figure 3B data. According to eq 2, the ratio of the slope to the intercept gives the association constant (K_a) as 1.69×10^3 M^{-1} for glucose, 3.33×10^3 M^{-1} for galactose, 1.61×10^3 M^{-1} for fucose, 4.06×10^3 M^{-1} for maltose and 1.85×10^3 M^{-1} for mannose. The association constant of the functionalized carbohydrates in the QCM response is larger in the order of fucose < glucose < mannose < galactose. Since maltose is disaccharide, it is not appropriate to compare the affinity of this disaccharide with those of monosaccharides. However, we confirmed that the small size

of the carbohydrate does not play significant roles to making the frequency change (figure 3A), the association constant for maltose, which showed the highest value among the carbohydrates, was over 2 times higher than that observed for fucose. It has been reported that the order of the higher association constants of the boronic acid for the carbohydrate molecule is glucose (110 M^{-1}) < mannose (172 M^{-1}) < galactose (276 M^{-1})[17,23]. The association constants for the carbohydrates in present study also exhibited a similar trend. The higher associate constant in our study confirmed the multivalent carbohydrate receptor and multiple carbohydrate moieties on AuNPs provide increased signal for the enhancement of carbohydrate detection. These results demonstrated that polyvalent interactions could be collectively much stronger than corresponding monovalent interactions. The different association constants can be explained from the structural point view of carbohydrates. It indicated that our synthesized boropolymer might be useful as selective carbohydrate agents for carbohydrate purifications.

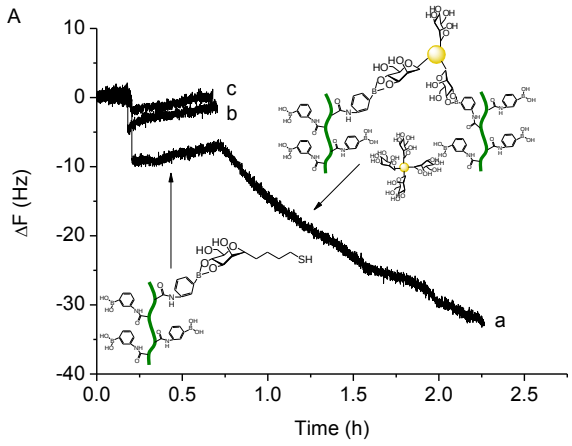

Figure 3. A. Frequency change vs time curves of boropolymer-cysteamine modified Au quartz exposed to 0.1 mg/mL mannose thiols and 20 μL AuNPs in series (a), cysteamine

modified Au quartz exposed to 0.1 mg/mL mannose thiols (b) and boropolymer - cysteamine modified Au quartz exposed to 20 µL AuNPs (c).

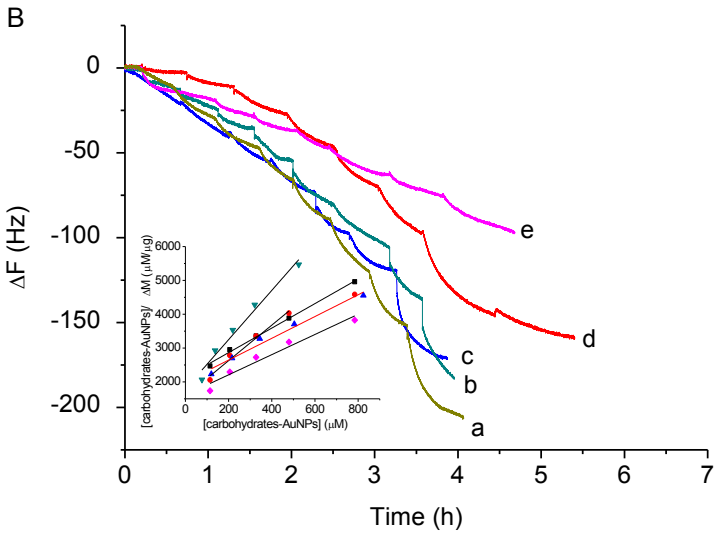

Figure 3. B. Frequency change vs time curves of boropolymer modified Au quartz electrodes exposed to mannose-AuNPs (a), fucose-AuNPs (b), galactose-AuNPs (c), glucose-AuNPs (d), and maltose-AuNPs (e). 2.5, 7.5, 17.5, 37.5, 67.5, 107.5, 157.5 and 257.5µg/mL carbohydrate-GNP were added in series. AuNPs size, 15nm. Inset, $[carbohydrates\text{-}AuNPs]_0$ /ΔM vs $[carbohydrates\text{-}AuNPs]_0$ of carbohydrates-AuNPs binding with boropolymer. maltose-AuNPs (▼), glucose-AuNPs (■), galactose-AuNPs (●), fucose-AuNPs (▲), mannose-AuNPs (◆).

Different size and shape of AuNPs have different molar mass as well as different surface areas so they have different abilities to conjugate carbohydrates. In order to understand the size and shape effect of AuNPs on the carbohydrate binding ability to the boropolymers, three different diameters of AuNPs (10 nm, 30 nm, 30 nm) and a different shape of Au nanorods were used to conjugate with carbohydrates respectively. Fig. 4 shows the frequency responses of the boropolymer modified Au quartz electrodes when different sizes and shape of AuNPs conjugated with mannose were added. It can be seen that that the amplification ability of AuNPs was heavily dependent upon their size and shape. 30 nm AuNPs has a highest ability to amplify the signal than that of others. This may be explained that the smaller size of 15 nm AuNPs has smaller surface area and has less number of carbohydrates immobilized. On the other hand, the bigger size of 50 nm AuNPs even though has more surface area and higher molar mass but will have smaller diffusion coefficient as well as the steric effect due to its big size which could prevent the mannose approach to boropolymer. Meanwhile, 30nm spheric AuNPs could more easily access the boropolymer layer compared with rod shape of Au nanorods. Thus 30nm AuNPs was chosen in the further studies of boropolymer carbohydrate biosensors.

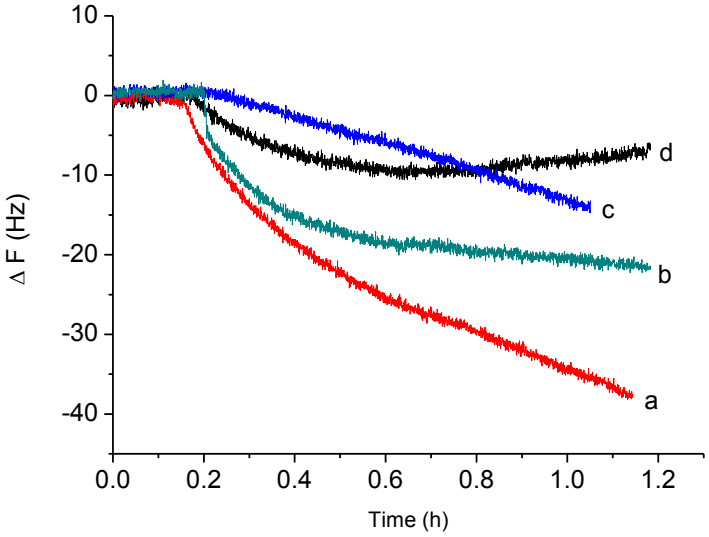

Figure 4. Size and shape of AuNPs effect on the interaction between boropolymer and mannose derivative. Mannose – 30 nm AuNPs (a), Mannose - Nanorods (b), Mannose – 50 nm AuNPs (c), Mannose-15 nm AuNPs (d). 15 µg/ml mannose-AuNPs was added.

Under optimized experimental conditions of AuNPs (spheric and 30 nm in size), quantification of mannose-conjugated AuNPs using the boropolymer sensor was carried out in a concentration range of 1.5 to 3825 µM (figure. 5). A good linear relationship in the mannose concentration ranging from 23 µM to 3.825 mM was obtained with a relative linear coefficient of 0.9852. The experimental determined detection limit of this sensor was 1.5 µM, which is much lower than that of using phenylboronic acid monolayer as recognition element but without oriented immobilization and amplification as we show in this report[35]. The multivalent carbohydrate receptor of boropolymer, the oriented immobilization of boropolymer via isourea bond formation, the stable and biologically active carbohydrate - AuNPs conjugates thus represent an innovative

approach for the high sensitivity and low detection limit of carbohydrate detection for glycobiology research.

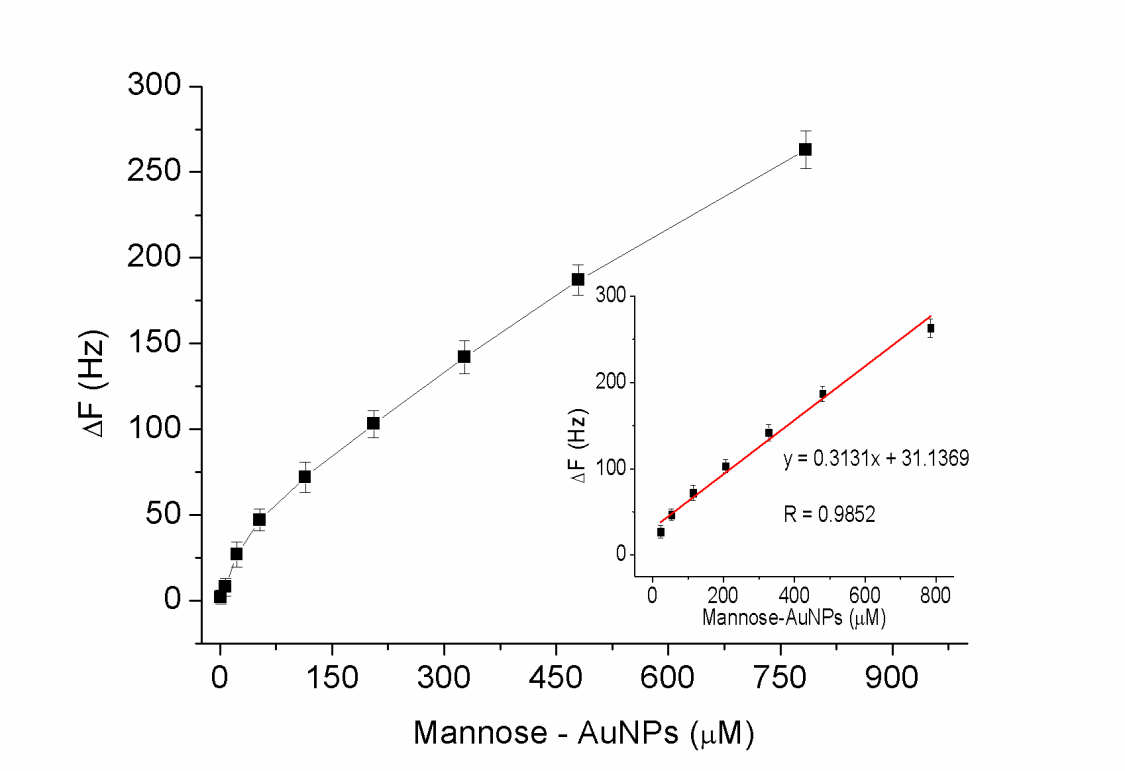

Figure 5. Calibration curve for mannose-AuNPs (30nm) obtained with boropolymer modified quartz surface. Inset is the linear relationship of the frequency vs the concentration of mannose (n = 3).

Carbohydrate - boronic acid complex formation is a pH - dependent reversible process. In present study, we investigated whether the reversible interaction can be used to regenerate the boropolymer recognition element on the sensor surface. As shown in figure 6A, The electron – transfer resistance R_{et} of the $Fe(CN)_6^{3-/4-}$ redox couple upon interaction with mannose-AuNPs (figure 6A, a) was found to decrease after regenerating the surface with 1 M acetic acid for 30 min (figure 6A, b), implying that mannose - AuNPs underwent a desorption process with boronic acid group. After reinjection of mannose - AuNPs to the cell, the electron - transfer resistance R_{et} almost recovered to its initial value(Figure 6A, c), indicating little loss of binding affinity of boropolymer towards mannose on the sensor surface. Moreover, the process of regeneration using acetic acid can be repeated (Figure 6A, d). The reproducibility of carbohydrates binding to the boropolymer monolayer monitored during three subsequently repeated experiments with the same concentration of mannose-AuNPs (10 μg/mL) was better than 84% via QCM detection methods (figure 6B). The proposed carbohydrate sensor could be used for at least 10 regeneration cycles without any significant decrease of sensitivity. The modified surface can be easily regenerated by dissociating the bond carbohydrates, previously captured by the immobilized boropolymer, using 1M acetic acid. Furthermore, the exposure to 1M acetic acid did not significantly affect the immobilized boropolymer, and did not reduce the ability of carbohydrate binding to boropolymer. This was attributed to the characteristic of boronic acid and the stabilized boropolymer structure. The reversibility and stability of the boropolymer based carbohydrate biosensor described in this work can greatly reduce the operating costs by allowing the electrode materials to be reused and the modified interface to be regenerated quickly and inexpensively.

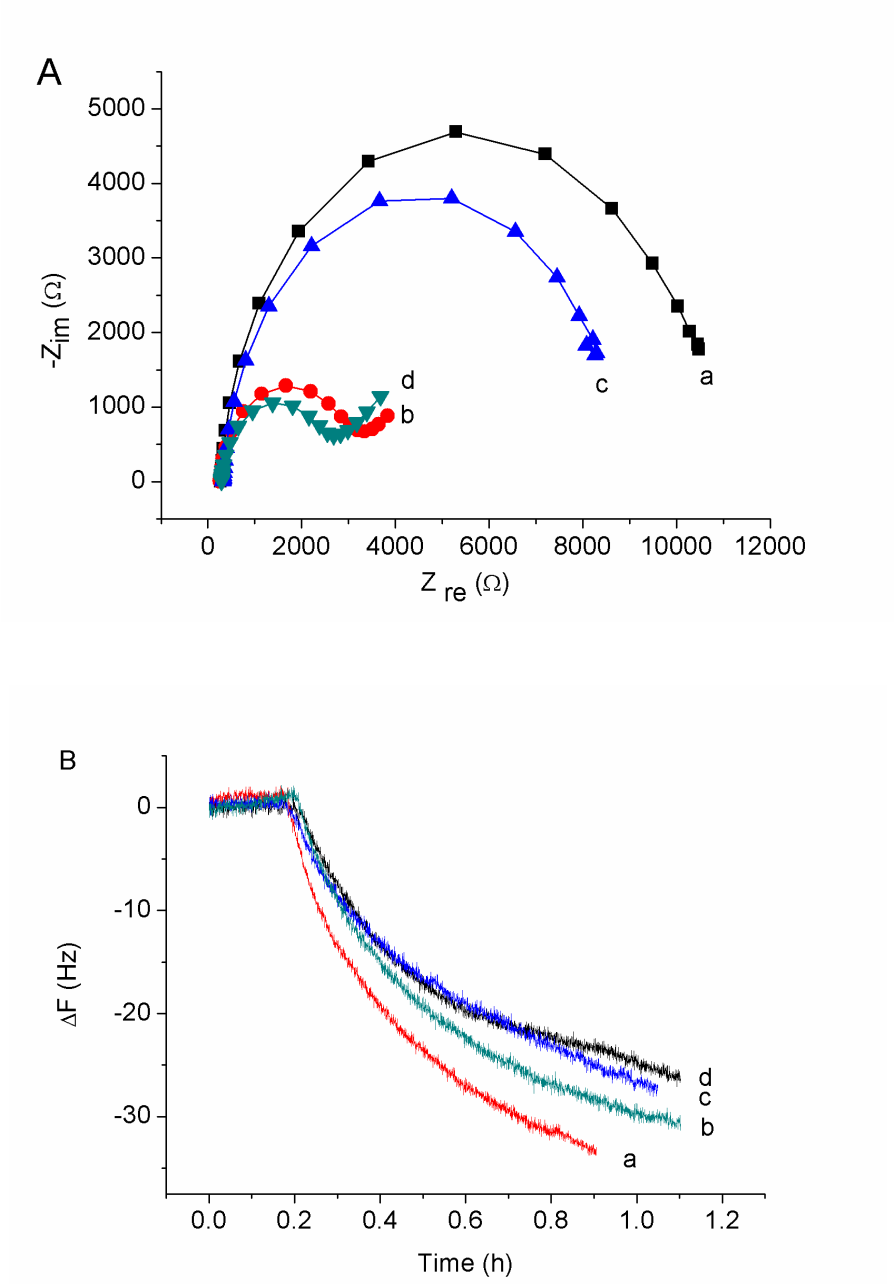

Figure 6. Reversibility of the boropolymer modified electrodes upon adsorption and desorption of mannose-AuNPs. (A) Complex impedance plots for boropolymer modified electrodes interaction with mannose-AuNPs (a, c), regeneration with acetic acid (b,d) in 5mM $Fe(CN)_6^{3-/4-}$ / 0.1 M KCl solution. (B) Frequency change vs time curves of

boropolymer modified electrodes exposed to 10μg/mL mannose-AuNPs. (a-d, regeneration boropolymer surface from 0 to 3 times)

4.4. Conclusions

Multivalent interaction between a boronic acid - containing polymer and AuNP - carbohydrate conjugates was explored for a sensitive and selective biosensor for studying carbohydrate - receptor interaction and carbohydrate detection. The boropolymer containing multivalent boronic acids attaching onto polyacrylamide backbone and *O*-cyanate chain end group which allows its oriented immobilization on the amine terminated SAM on Au electrode. Cyclic voltammetry, electrochemical impedance spectroscopy were used to characterize the sequential assembly of the boropolymers on the Au sensor surface and QCM was used to illustrate the performance properties of the resulting boropolymer immobilized sensor for the carbohydrate detection. The multivalent boronic acids on boropolymer increased its binding sites with the multivalent carbohydrates on the AuNPs thus allow significant increase of the response signal. We compared different size and shape of AuNPs and found 30 nm AuNPs gave the best results as a carbohydrate carrier for signal amplification. We studied the binding between five carbohydrate conjugated AuNPs with the boropolymer. In our results, the interaction between the boropolymer on the sensor chip and the monosaccharides was in the order fucose < glucose < mannose < galactose. Disaccharide maltose showed the highest sensitivity among the carbohydrates. The boropolymer based carbohydrate sensor gave a linear response to mannose in the range from 23 μM to 3.825 mM and a lower detection limit of 1.5 μM. The reversible binding between boropolymer and carbohydrates allow the regeneration of the ligands. The results revealed that the sensor can be regenerated

using 1 M acetic acid without significant effect on the immobilized boropolymers. The greater progress achieved through this study will aid the effort to develop glyco-sensor and will enhance the potential for future discoveries in the fields of carbohydrate research and carbohydrate analysis. The study of polyvalent interaction may provide new targets and new strategies for the design of pharmaceutical agents.

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CHAPTER V

SUMMARY

In this thesis study, oriented and multivalent carbohydrate-binding macromolecules were designed and developed based on a chain-end functionalized boronic acid-containing polymer (boropolymer) for glyco-capturing, glycomics and glycoproteomics applications.

First, a biotin chain-end functionalized boropolymer was synthesized *via* arylamine initiated cyanoxyl-mediated free radical polymerization as a functional glyco-affinity macroligand. The specific streptavidin binding capacity of biotin boropolymer was confirmed by streptavidin-HABA assay, while Alizarin Red S binding assay evaluated the specific carbohydrate binding capacity of biotin boropolymer. A strategy for oriented immobilization of boropolymer onto magnetic bead surface through specific streptavidin-biotin binding and its glyco-affinity capturing and direct MALDI-MS identification were demonstrated. It is a unique advantage to use magnetic beads and chain-end functionalized boropolymer as a glyco-affinity macroligand for efficient purification or enrichment of carbohydrates and glycocojugates.

Second, an *O*-cyanate chain end functionalized boronic acids containing polymer has been designed and synthesized in one-pot fashion. The synthesis is straightforward without protection/deprotection and subsequent conversion needed. A strategy for

oriented boropolymers immobilization onto amine surface through isourea bond formation and its carbohydrate and glycoconjugate capturing were confirmed by both QCM and AFM techniques. The immobilization was performed in mild aqueous condition. The resultant 3D carbohydrate receptor will find important application for efficient carbohydrate sensoring with enhanced selectivity and affinity. On the other hand, because the amine-grafted surface can be assembled on various supports such as gold, glass, silica, or alumina, presented oriented multivalent carbohydrate receptor could be extended to many support materials and for a variety application. For example, this glyco-capturing approach can be used for carbohydrates and glycoproteins as well as cells isolation purification application.

Third, we studied the multivalent interactions of the immobilized *O*-Cyanate chain end functionalized boropolymer with five different carbohydrate conjugated AuNPs. Our studies showed that different carbohydrates have different binding constants. Furthermore, the multivalent binding between carbohydrates and boropolymer was reversible and allowed the regeneration of boropolymer surface for sequential capture and release of the carbohydrate analytes.

This oriented multivalent glyco-affinity ligands could be used for efficient carbohydrate and glyco-conjugates purification and identification and thus is expected to constitute a core strategy of glycomics and glycoproteomics targeting glyco-proteins.

CHAPTER VI

FUTURE PERSPECTIVES

Overall, in this study, oriented multivalent glyco-affinity ligands have been developed and could be used for efficient carbohydrate and glyco-conjugates purification and identification and thus are expected to constitute a core strategy of glycomics and glycoproteomics targeting glyco-proteins. It is well known that structural alteration of glycans is recognized to be associated with cell differentiation and certain disease states. Glycans can be used as a target molecule for biomarker discovery. In particular, sialoglycans, which have sialic acids at their terminal end, are one of the major classes of glycans used for biomarker discovery

6.1. Serum sialic acids are biomakers of cardiovascular disease

Sialic acid (SA) is a *N*-acetylated derivative of neuraminic acid that is an abundant terminal monosaccharide of glycoconjugates such as glycoproteins and glycolipids. SA residues on cell surface are antigenic determinant residues in carbohydrate chains and are involved in signal recognition and adhesion to ligands, antibodies, enzymes and microbes[1]. Normal human serum SA is largely bound to glycoproteins or glycolipids (Total sialic a1.5–2.5 mmol/L), with small amounts of free SA (1–3 μmol/L)[2]. Substantial evidence now shows that serum sialic acid (SA) is a possible risk factor for CVD. The concentration of serum total sialic acid (TSA) is related to the risk factors of cardiovascular diseases[3]. In the people suffering from diabetes the level of TSA increased and increased risk of cardiovascular diseases was observed in these patients[4]. Several studies have shown that the level of TSA increases in the people afflicted by CVD[5]. The

level of sialic acid as a predictor index in long periods for coronary heart diseases (CHD) (especially for women) that lack any cardiovascular diseases was suggested[6]. Another study showed that the level of TSA increases in proportion with the numbers of the coronary artery diseases (attack times) and the concentration of lipid-bound associated sialic acid was related to the severity of coronary atherosclerosis especially in patients with two or three times vascular disease[7]. There are evidences showing that the mean of TSA in people afflicted with acute myocardial infarction (AMI) increased significantly compared to the control group indicating that TSA is a risk factor for coronary artery diseases[8,9]. The significance of SA as a novel marker for cariovascular disease has been recently reviewed[10].

Due to the great significance of SA in physiological, clinical, and pharmaceutical applications, several analytical methods have been developed for SA measurements[10]. These include colorimetric[11-13], fluorometric[14,15], and chromatographic methods[16-18]. Several schemes have been suggested as well for the assay of SA based on a sequence of two or more enzymes with either colorimetric or fluorometric finish. However, current measurements of sialic acids suffer both from sensitivity and efficiency, especially are not adequate to acute cardiovascular disease testing[10]. Therefore, there is apparent need to develop a sensitive and efficient method to determine sialic acid. On the other hand, all current efforts focus on determination of sialic acid hydrolyzed from the sialyl conjugates in the serum and free sialic acid circulating in the serum. It will be very important to determine the sialyl conjugates in serum, which will be the accurate biomarkers of cardiovascular diseases.

6.2. Functional Glyco-Affinity Marcroligand for Sialic Acids Biomarkers identification

In future research, we will seek to define the selective capturing capacity of chain-end functionalized boropolymer for free sialic acid. As mentioned in the introduction, boronate ions form complex with diols in carbohydrate in various constants varying from 6 to 2000 M^{-1} for different sugars[19]. This suggests high selectivity of boronate interaction with carbohydrates. Ostuka, *et al*. evaluated the equilibrium constant (K_{ass}) for sialic acid binding to 3-(propionamido) phenylboronic acid (PAPBA) by comparing with those for glucose, mannose, and galactose[20]. They found that the boronic acid selectively recognizes sialic acid at physiological pH**.** In the sialic acid/PAPBA system, the formation of a trigonal-formed complex stabilized by the coordination of an amide group of sialic acid at the C-5 position to the boron atom, forming intramolecular B-N or B-O bonding, supported the high complexing ability of boronate with sialic acid at physiological pH 7.4. So, in our next study, we intend to investigate the selective capturing capacity of chain-end functionalized boropolymer for free sialic acid.

A few methods for sialyl-protein isolation have been published including chemical derivatization using hydrazine chemistry[21,22], which captures both SA and neutral glycosylated peptide**s**/proteins, or the use of glycan-binding proteins, lectins[23-25]. There are examples of lectins that are specific for SA including Sambucus nigra agglutinin, Maackia amurensis leukoagglutinin I/II, and the Siglecs family, but most lectin chromatography suffers from rather broad specificities and co-purification of non-glycosylated proteins/peptide**s**. Most recently, titanium dioxide (TiO_2) particles has been used to capture sialyl-proteins through the complex formation between the negative charge of the carboxylate of sialic acid and the titanium dioxide[26]. However, other

negative charge containing molecules such as sulfates and carboxylates-containing carbohydrates or proteins might also be co-captured. Therefore, presently there is no efficient and simple method for the selective purification of sialyl-proteins from complex biofluids such as plasma or serum. Therefore, in our further studies we intend to investigate the selective capturing capacity of chain-end functionalized boropolymer for sialyl-protein.

6.3 References

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